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Susceptibility to desiccation and soil factors affecting the survival of Rhizobium japonicum strains

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Susceptibility to desiccation and soil factors affecting the survival of *Rhizobium japonicum* strains

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INTRODUCTION

Soybean (Glycine max L. Merr.) production is expanding rapidly throughout the world as a result of the high demand for the grain as an excellent source of oil and protein. For a long period the United States and China were the largest producers, far ahead of the other countries. However, soybean production recently in many countries has been expanding rapidly.

In 20 years (1955-1975), the world area planted to soybeans has expanded from 80,000 to 8 million hectares. Considering an average of 70 kg of nitrogen fixed per hectare by symbiosis, an annual savings of 560,000 tons of nitrogen taken from the air instead of from the soil or fertilizer can be realized.

Success in obtaining high N_2 fixation through the symbiosis of Rhizobium japonicum with soybeans depends on a series of factors which may interact increasing the complexity of the system: (a) effectiveness and efficiency of the R. japonicum strains present in the inoculum and/or soil in relation to soybean varieties and the environment; (b) numbers of viable rhizobia cells in the inoculum compared to the native population of R. japonicum; (c) techniques of inoculation and seeding to provide adequate survival and multiplication of rhizobia around the developing roots; (d) environmental factors, mainly soil factors, which will affect survival of the intro-

duced rhizobia in soil, their establishment, rapid nodule initiation and/or functioning, and the physiology of the plant.

The success of each stage is influenced to a considerable degree by the soil conditions surrounding the roots of the legume seedling. If soil conditions are unfavorable for the survival and growth of rhizobia prior to infection of the root hair, an effective nodule symbiosis will not develop.

Rhizobia, like all other Gram-negative, nonspore forming bacteria, are more sensitive to certain environmental soil conditions than their host counterpart. Therefore, more information is needed about the survival of different R. japonicum strains in different soils and the factors affecting their survival and adaptation.

The purpose of this study was to evaluate the survival of different R. japonicum strains in saline soils as developed in a hot dry climate. More specifically, the objectives were to:

1. Study the survival of two Australian and two Beltsville R. japonicum strains in soils of different texture and organic matter contents.
2. Study the effect of temperature, salt concentration, and soil moisture content on the survival of different R. japonicum strains.
3. Examine the differences between the strains in terms of bacterial water relationships.

4. Study if the degree of survival of these strains can be improved by reducing the internal water content of the bacteria at low relative vapor pressure.

REVIEW OF LITERATURE

Factors Affecting Survival and Growth of Rhizobia in Soils

Soil factors

Since the persistence of the rhizobia in soil is of importance for the cultivation of legumes, it has been the subject of many studies. The survival and growth of root-nodule bacterial groups vary in different soils under a variety of environmental influences. Some of the soil characteristics affecting the survival and growth of rhizobia will be discussed separately. It is realized that only rarely can a factor be considered in isolation; normally interaction occurs between a given factor and the other factors of the environment.

Soil moisture Moisture content of the soil will affect the activity of rhizobia in two ways. First, water is a major component of protoplasm, and second, an adequate layer of external water is necessary for maintenance of turgor and movement of substrates, toxic products, cells, etc. Therefore, as a soil dries and the soil moisture tension becomes greater, growth of rhizobia would decline and death would result.

Fred et al. (1932) early reported on the loss of viability of rhizobia on the surface of inoculated seed. In a detailed study, Vincent et al. (1962) recognized two phases under drying conditions. The death rate was greatest during the first few hours of desiccation (approximately 24 hours) when the

main loss of water was occurring, and a storage period in which the death rate was reduced. They found that R. trifolii lost viability when suspended in distilled water and spread on glass beads under both drying (0 and 20% R.H.) and non-drying (100% R.H.) conditions. Suspension in 9% maltose appeared to increase the survival of R. trifolii under desiccating conditions. Survival of R. meliloti in soil culture was studied by Jensen (1961). He reported survival in dry storage of up to $5x10^5$ cells/g soil after 30 to 40 years in a medium containing sterilized loamy garden soil, calcium carbonate, and 5% mannitol. Since the number of cells originally present was unknown, one must assume that a considerable loss had occurred. Vincent (1965) interpreted the survival of those cells under desiccating soil conditions as being due to either a chance and/or a metabolic inertness, which enable the bacteria to survive the critical drying period after which they were somewhat resistant to death.

The effect of desiccation on the survival of rhizobia has important implications in the seeding of inoculated pasture legumes either into a dry seedbed or broadcasted onto the soil surface. It would be expected that rhizobia adhering to the surface of a legume seed would be subject to desiccation when sown into a dry soil. Studies by Alexander and Chamblee (1965) have shown that the effectiveness of inoculation was reduced when inoculated alfalfa and birdsfoot trefoil were seeded into a soil dried below the wilting point. Exposure

to sunlight when placed on a dry soil surface for 3 hours or more likewise reduced inoculation effectiveness, and this was probably the result of desiccation rather than the effect of the radiation on the bacterial cells. According to Stanier et al. (1963), visible light has a negligible lethal effect on bacteria because their essential chemical components absorb little or no light in the visible region.

Recently, Davidson and Reuszer (1978) studied the persistence of R. japonicum strain 61A68 when added to surface-sterilized soybean seeds along with 12 different coating materials under controlled temperature and moisture. They found with various coatings from 0.9 to 19.3% of the original inoculum survived for 3 weeks at a storage temperature of 15°C and 50 ± 5% relative humidity. At 22.5°C, from 0.5 to 7.2% of the original inoculum survived, and at 30°C, from 0.1 to 1.6% of the original inoculum survived. The data indicated that extremely large numbers of R. japonicum would have to be added to the seed to have adequate numbers survive for nodulation following 3 weeks of storage at ordinary temperatures.

Soil texture The effects of soil texture on survival and growth of rhizobia are indirect, resulting from the physical and chemical characteristics they confer on soil.

Survival of R. japonicum in stored air-dry soils was evaluated by Abhiswar and Arindra (1956). Nine samples of surface soil containing viable R. japonicum were collected in soybean fields from different parts of India. After 19 years

of storage in an air-dry condition, only two soils (Akola and Jorhat) contained viable R. japonicum. The texture of the Akola soil was clay and of the Jorhat soil was loamy sand. The authors did not offer any evidence for the longevity of the rhizobia in Akola and Jorhat soils; however, they suggested that the survival of the rhizobia may be related to the presence of organic substances or of trace elements in the soil solution.

Damirgi (1963) reported that R. japonicum serogroups 3, 31, and 123 were present in soybean nodules in all areas of Iowa that were tested, but the frequency of those serogroups varied among soil types.

In extensive areas of the agricultural region of western Australia, clover root-nodule bacteria apparently fail to survive into the second growing season despite the excellent nodulation obtained in the establishment year as a result of seed inoculation (Marshall et al., 1963). They found with most probable number counts, in several problem soils during the second growing season, that the number was less than 10 bacteria per gram of soil compared with 10,000 to 100,000 in nonproblem soils. The results were related to soil conditions. They found severe mortality on gray sand in the second season with more than 80% of the plants not nodulated. Mortality was less severe, with 50 to 70%, on yellow sandy soils which contained small amounts of finer material. There was no measurable mortality associated with the red sandy soils contain-

ing 6 to 8% silt plus clay. Other finer textured soils also showed no obvious mortality. The authors suggested that there was a relationship incidence of the condition and physical soil properties, particularly texture.

Marshall and Roberts (1963) studied the influence of amendments on survival of R. trifolii in sandy soils. They found that the addition of fly ash or a montmorillonitic clay to a sandy soil improved the percentage of clover nodulation in the second growing season. They suggested that these materials might protect the root-nodule bacteria from death during the extremely hot, dry summer. In laboratory experiments, Marshall (1964) found that fly ash or montmorillonite amendment of sandy soil protected R. trifolii from the adverse effects of drying and exposure to high temperature. Their results showed that the survival in the amended soils exposed to high temperatures, 50 and 70°C, was significantly greater than in the unamended soils.

Ham (1967) studied the correlation of R. japonicum serogroups with soil properties. In the two-year study, the correlation of R. japonicum serogroups 3 and 31 in soybean nodules was negatively correlated with the sand percent of the soil from which the nodules were taken. Serogroups 3 and 31 were positively correlated with silt percent in both years.

Soil water retention is influenced by particle composition of the soil. Bouyoucos and Cook (1967) measured the relative humidity of soils at different moisture contents by the

gray hydrocal hygrometer. They found that the addition of 2% water to 40 g oven-dry fine sand resulted in a relative humidity of 99.7% and a soil water tension of 4.0 atm; when added to an oven-dry sandy loam, the corresponding values were 64.5 and 589 atm; when added to a clay loam, the values were 30% and 1617 atm; and when added to a silty clay loam, the values were 10% and 3093 atm. Water content of soil, however, has been shown to markedly influence the microbial composition in the soil (Stotzky, 1974).

Weaver et al. (1972) found a wide range in counts of R. japonicum (from less than 10 to more than 10^6 rhizobia per g of soil) in soils at 52 sites in Iowa. Numbers were related principally to cropping history, soil texture, and organic matter contents.

Danso and Alexander (1974) studied the survival of R. meliloti strain M_3V_2-S in two soils and in sand. Soils were Valois silt loam, pH 5.6, and Adams-Calton loamy fine sand, pH 5.0. Water or an inoculum was added to bring the moisture content of the soils to 22% (Valois), 14% (Adams-Calton), and 30% (sand) on a dry-weight basis. The data revealed no significant decline at these moisture levels in number of viable cells during the 8-week test period in the sand or in the two soils. However, under drying conditions over calcium chloride in a desiccator, a difference in survivability was noted between the two nonsterilized soils and a sample of sterilized sand. Results, under drying conditions, showed

that the number of viable cells in the sterilized sand fell very rapidly and less than 10 cells per g were found at 4 weeks. By contrast, the R. meliloti survived in the desiccated soils.

Ionic composition The soil solution is a weak electrolyte composed of a variety of organic and inorganic cations and anions of different valencies. Microbial cell membranes and many soil particles interact with these ions.

Day (1942) showed that the moisture potential of the soil is the summation of (a) the pressure potential (free energy per unit mass developed by attractive forces between soil particles and surrounding water), and (b) the osmotic potential (free energy per unit mass due to solutes).

Brown (1964) discussed the bacterial response to the ionic environment. He explained the roles of osmotic pressure and water activity as a mechanism of ionic effect on the bacterial cell. He concluded that the ionic effect on bacteria is directly through the ions themselves. The effects of water activity and osmotic pressure, in many cases, are probably of minor importance.

The influences of various salts such as chlorides, nitrates, and sulfates on the viability of rhizobia has been studied by many investigators. Wilson (1917), exposing soybean bacteria to different salts of various concentrations and combinations for 4 to 6 weeks, found that nodules were produced in 61 out of 77 cases. The failure of the other 16 cases to

form nodules was due to injuries caused by the high salt concentrations and unsuitable salt combinations. In another experiment employing soil, he found that the presence of high amounts of nitrate and sulfate prevented nodule formation. However, the bacteria were still alive and were capable of forming nodules when introduced to more favorable environmental conditions. Ohkawara (1928) found that tested strains of root-nodule bacteria taken from various legumes had retained their viability for more than 40 days in 0.1% potassium nitrate, sodium nitrate, and calcium nitrate, but not in the same concentration of ammonium sulfate. Hill (1918) found that approximately 25 mg of nitrate in 100 g of soil actually stimulated the multiplication of R. trifolii. Higher concentrations of nitrate had a depressing effect. Furthermore, 100 to 150 mg of nitrate per 100 g soil appeared to be toxic.

In a soil which would not support growth of clover rhizobia, Vincent and Waters (1954) found a population of $3x10^8$ to $5x10^8$ per g of soil within 7 days after adding calcium hydroxide. Norris (1958) showed that the application of lime stimulated bacterial growth either by neutralizing the acidity or by supplying the needed divalent calcium. He found that when acidity was eliminated by sodium, potassium, and ammonium hydroxides no growth occurred. But when magnesium or barium hydroxides were applied, their effects were similar to calcium in stimulating the growth. These results suggested that calcium is not an essential factor for the

bacterial growth. However, he pointed out that a divalent cation was needed. Bezdicek (1972) found a positive correlation between R. japonicum serogroup 123 and soil magnesium. Other serogroups had no significant effect.

Wilson and Norris (1970) measured the growth of R. japonicum strain CB 756 in pure culture at four levels of sodium chloride, 0, 40, 80 and 160 meq per liter. Their results showed that the multiplication of strain CB 756 was not affected by a salt concentration of 40 meq sodium chloride per liter of broth but was reduced at 80 and completely inhibited at 160 meq sodium chloride per liter. They explained that the long lag period at 80 meq was due to either (1) a small population of salt tolerant cells present in the original inoculant, or (2) the strain adapted to grow at this level of salt.

Temperature Rhizobia, like all nonspore forming bacteria, are sensitive to environmental changes. In the soil, they are exposed to several factors which may affect their density. Of these, soil temperature may be one of the most important.

Alicante (1926) found that Bacillus radicicola (previous name for Rhizobium spp.) of cowpea and soybean was killed after 15-minute exposure to 50°C in solution. In another experiment, he studied the effect of different soil types upon the thermal death point of legume organisms. His results showed that B. radicicola of garden peas was alive in peat after a 10-minute exposure to 50°C. In the second determination, all organisms

in the brown silt loam were dead.

Meyer and Anderson (1959) studied the effects of temperature, inoculation with rhizobia, and potassium nitrate on the yield and protein percentage of subterranean clover. Plants were grown on nitrogen-free nutrient agar in test tubes placed in a temperature controlled water bath in the glasshouse. The results showed that the uninoculated plants responded similarly to added nitrogen at temperatures of 20 and 30°C. By contrast, plants inoculated with nitrogen fixing Rhizobium spp. grew normally only at 20°C. Yields expressed as mg dry matter per plant were 51.8 and 62.0 at 20 and 30°C, respectively, when potassium nitrate was added; however, when inoculated with Rhizobium spp., the yields were 54.5 and 35.5 mg, respectively. Gibson (1961), by exposing subterranean clover roots to different temperatures, showed that the effectiveness of nitrogen fixation by strain NA 30 on two varieties of clover was reduced considerably at temperatures above 22°C. However, the effectiveness of strain TA 1 remained comparatively constant at higher temperatures. Their results with strain NA 30 were similar to those reported by Meyer and Anderson (1959).

Nodule formation (infectiveness) may be reduced at moderately elevated temperatures. The degree of inhibition is dependent on the host plant and the rhizobial strain (Pate, 1961). Pate studied the effect of temperature on the nodulation process by selected strains of rhizobia on barrel medick

(Medicago tribuloides) and purple vetch (Vicia atropurpurea). Each host-bacterium combination was grown in nitrogen-free sand cultures in a series of constant temperatures (6-30°C). All nodulation curves showed optima at or near the temperature extremes of growth. He found that strains V33 and L2 were particularly effective in forming nodules at high temperature, while other bacterial strains (M5 and V5) demonstrated the opposite effect.

In a field experiment with inoculated clover seed, Marshall and Robert (1963) examined three fine particulate materials as soil amendments: finely ground silica, a montmorillonite clay, and fly ash. Nodulation counts during the growing season showed that 73% of the plants were nodulated with fly ash, 54% with clay, and 21% with the fine silica. Nodulation in the control treatment was 28%. They suggested that these materials might protect the root-nodulation bacteria from death during dry summers. Marshall (1964) found that the amendment of sand with fly ash or montmorillonite was helpful in protecting R. trifolii from the adverse effects of drying and exposure to high temperatures. He indicated also that the fast-growing species, such as R. meliloti and R. trifolii, failed to survive at 70°C in dry sandy soil. However, those species were able to form nodules at 70°C when montmorillonite was added to the sandy soils. The slow-growing species, R. lupini and R. japonicum, were markedly resistant to the drying condition. Montmorillonite increased soybean rhizobia (R.

japonicum strain QA 372) in the uninoculated soils from 6.23 to 6.77 (log viable cells per g soil) at nil heat temperature, from 6.08 to 6.31 at 50°C, and from 4.34 to 4.77 at 70°C.

Biological factors

Cell morphology Size and shape of bacterial cells reflect the prevailing environmental conditions. According to Lamanna and Mallette (1965), zoologists have long recognized Bergmann's rule which states that the body sizes of races living in cooler climates are larger than those races of the same species living in warmer zones. This rule has been found applicable to both vertebrates and invertebrates.

The relationship between temperature and size in the genus Bacillus was studied by Lamanna (1940). Body size of the organisms was measured with a filar micrometer. He pointed out that these organisms incubated at 45 and 50°C were usually smaller than those isolated from materials kept at room temperature. He measured the sizes of B. vulgatus and B. mesentericus at two different temperatures, 50-55 and 55-60°C. Results, for example, with B. vulgatus showed that the cell size was 0.56x2.33 µm in the temperature range 55-60°C, compared with 0.68x2.80 µm in the 50-55°C range. In other comparisons between species of Bacillus, the cell size was also lower with species incubated at higher temperature. He found with B. subtilis, incubated at 55-60°C, the cell size was 0.62x2.27 µm (average of 16 cultures) in contrast with B. cereus, incu-

bated at 40-50°C, the cell size was 1.00x3.60 μm (average of 20 cultures). He also found with 34 other cultures of B. megaterium and B. mycoides that cultures adapted for growth at lower temperature were larger in size than similar cultures adapted for maximum growth at higher temperature.

Lamanna and Mallette (1965) discussed cell morphology alteration in conditions of desiccation and reduced nutrition. They pointed out that under conditions of desiccation, the cells decreased their surface to volume ratio and became more spherical. Under condition of reduced nutrition, the surface to volume ratio increased and cells became elongated.

Based on a variety of direct microscopic observations, Casida (1971) found that the dormant bacterial cells of unamended soil appeared to be composed mainly of coccoid and coccoid-rod cells, ranging in diameter from 0.5 to 0.8 μm and covered with a capsule-like material.

Cell water relations Not all the water in a medium is free and available to bacteria. Many water molecules are associated with solute molecules. This association results in a lower moisture tension for the solution than for pure water.

Christian and Waltho (1964) studied the composition of Staphylococcus aureus in relation to the water activity (A_w) of the growth medium. They found that cell water decreased from 1.66 to 0.63 g H_2O per g cell dry weight when the A_w decreased from 0.993 to 0.900. Internal concentrations of solutes generally increased with decreases in A_w.

Chen and Alexander (1973) reported a general relationship between desiccation resistance of soil bacteria and their ability to grow in media of reduced water activity. A comparison was made of the survivability of bacteria grown in basal medium and in the same medium adjusted with salts to the appropriate level for the minimum growth of the orgnism. The minimum A_w values allowing growth of their five drought-susceptible bacteria were 0.985, 0.960, 0.995, 0.990, and 0.985 for Flavobacterium sp., Pseudomonas sp., Rhizobium sp., and Gram-negative rods S_3 and S_5, respectively. Growth of all five bacteria in media with high salt levels allowed the isolates to survive longer in dry conditions. On the other hand, Boylen (1973) was unable to select species of Arthrobacter sp. resistance to desiccation. Also, Bushby and Marshall (1977a) were not successful in selecting desiccation resistant species of rhizobia by growth at low A_w.

From studies of bacterial adsorption isotherms (Bushby and Marshall, 1977b), there was a direct correlation between the amount of water retained by desiccated rhizobia and their ability to withstand desiccation. They found that greater amounts of water were retained at lower relative pressures by the desiccation-sensitive R. leguminosarum group than by the more resistant slow-growing rhizobia.

Cell surface properties The surface structure of bacteria has a variety of properties, such as (1) the ability to wet by various liquids and (2) electrical charge.

Marshall (1967) suggested that the surfaces of the slow-growing root-nodule bacteria contain only acidic (carboxyl) groups, while the fast-growing strains contain some base (amino) groups along with the predominant acid groups. Marshall (1969) found that rhizobia with a simple carboxyl surface adsorbed nearly twice the weight of montmorillonite or illite per unit area of rhizobial surface than rhizobia with a carboxyl plus amino surface. His results showed that R. lupini UT12 (carboxyl surface) adsorbed 4.70×10^{-6} µg montmorillonite per rhizobia, while R. trifolii SU297B and R. trifolii 298D (carboxyl plus amino surfaces) adsorbed 1.55×10^{-6} and 2.03×10^{-6} µg per rhizobia, respectively. He also found differences in adsorption within the same species; R. trifolii TA1 (carboxyl surface) adsorbed 3.98×10^{-6} µg montmorillonite per rhizobia. It appears that the nature of the ionogenic surfaces of root-nodule bacteria was the most important factor in determining the amount of montmorillonite or illite adsorbed. The surface area was 9.24, 6.82, 5.58, and 8.17 μm^2 for R. lupini UT12, R. trifolii TA1, R. trifolii SU297B, and R. trifolii SU298D, respectively. Marshall concluded that adsorption seemed to be independent of the net surface-charge density of either the rhizobia or the clay.

Ecological adaptation Both Gram negative and positive bacteria grow naturally in habitats of high osmotic pressure, such as sea water, salt lakes, and fruit juices. Furthermore, media of high osmotic concentrations may be needed for their

successful isolation in the laboratory. Most of these species can be adapted to grow in media of lower osmotic concentrations, while others are obligately osmophilic (Lamanna and Mallette, 1965).

Ecological adaptation of rhizobia within the same species was observed when strains of R. trifolii from America, Finland, and Holland were found to be less resistant to high temperatures in most soils than South African strains (Anon., 1958). Marshall (1964) found two species of native rhizobia, R. lupini and R. japonicum, to be better able to survive heating than R. meliloti in some Australian soils.

Ecological adaptation within rhizobial groups also has been reported by Wilkins (1967). The groups of rhizobia tested were those producing nodules on the genera Medicago, Psoralea, Lotus, and Acacia. Results for Acacia, Lotus, and Psoralea rhizobia suggested ecological adaptation within these groups from different climatic regions. The strains from the colder New England Tableland had a much lower resistance to high temperature than those from western New South Wales. Rhizobia survived in all air-dry soils exposed to 80°C for 5 hours. However, only two samples from western New South Wales survived exposure at 70°C, and one from New England survived exposure at 60°C. In moist soil, the temperature for rhizobial survival was lower than in air-dry soils. Sufficient sterile water was added to air-dry soils to produce a soil moisture tension of 30 mm Hg. Medic rhizobia and Psoralea rhizobia survived 5

hours at 50 and 60°C in moist soil, respectively. However, in air-dry soil, both survived a similar exposure at 100°C. Stotzky (1974) reported that, in pure cultures, cells and spores are more resistant to elevated temperatures when the water is low (e.g., the heat resistance of spores is greatest between an A_w of 0.40 and 0.22).

In New Zealand, Dye and Hastings (1969) selected strains of R. trifolii resistant to desiccation. They showed that some strains of R. trifolii collected from dry areas have much greater resistance to drying than those from areas where the soils remain moist for the greater part of the year.

Production of extracellular polysaccharides Many soil bacteria synthesize extracellular polysaccharides which diffuse away from the cells into the liquid medium, or from a mucoid layer around organisms growing on the surface of solid medium. Wilkinson (1958) pointed out that polysaccharides are hygroscopic and may allow the cells to rehydrate slowly after desiccation.

Markovitz and Sylven (1962) studied the effect of sodium sulfate and magnesium sulfate on heteropolysaccharide synthesis in Gram-negative soil bacteria. They found that the 21 strains studied could be divided into three groups on the basis of salt effects on polysaccharide composition, synthesis, or both.

Casida (1971) observed, by direct microscopy and staining, the occurrence of capsule-like structures surrounding bacteria in unamended soil. These structures, however, were apparently

dissimilar biochemically to capsules produced in laboratory cultures. Stotzky (1974) pointed out that mucoid surface layers impart adhesive properties to cells in soil; these properties may enhance attachment to soil particles and the formation of soil aggregates.

Polysaccharides are highly hydrated (Walker, 1975) and may protect the bacterial cells during conditions of water stress. The water present in the polysaccharides may be utilized directly by the bacteria. But Bushby and Marshall (1977a) found that the production of extracellular polysaccharides by R. trifolii strains SU 297/31A and SU 298/334C did not improve their survival in desiccated sandy soil.

Microbiology of Irradiated Soils

Soil can be sterilized with dry heat, steam, or chemicals. However, all three methods alter the chemical properties of the soil. Steam and heat cause many changes in the organic matter, and may also alter the nature of the mineral fraction. Some chemical sterilizers leave residues. For example, methyl bromide may leave a bromide residue which is toxic to certain plants (Eno and Popenoe, 1964). Salonius et al. (1967) compared the growth of Arthrobacter globiforms and Pseudomonas fluorescens in autoclaved and irradiated soils. They found higher population in the irradiated soil than in the autoclaved soil. They also observed that autoclaved soil contained more soluble organic matter, soluble carbohydrates, and water-

exchangeable electrolyte than the irradiated soil.

Survival of Microorganisms in Irradiated Soils

McLaren et al. (1957) and Eno and Popenoe (1964) pointed out that 2.5 Mrad is capable of sterilizing many soils. McLaren et al. (1962) did not detect viable organisms in three soils treated with 4 Mrad, and when Dublin clay loam soil was exposed to 1 Mrad, the survival of aerobic bacteria and actinomycetes was only 0.001%. Boyer et al. (1966) measured the viable microflora of 15 soils from Europe and Africa before and after treatment with 4 Mrad; they reported 0.0013% survival in the soil possessing the highest initial population, equal to 8.1×10^6 total microflora per gram soil.

The effect of different soil moisture levels on gamma radiation of microorganisms has been studied by numerous investigators. Popenoe and Eno (1962) obtained 4% survival of both fungi and bacteria plus actinomycetes in a fine sand irradiated with 1,024 Krad of gamma radiation at 10% moisture. In further experiments at 10% moisture, they obtained less than 3% survival of fungi and bacteria plus actinomycetes at a dose of 500 Krad of gamma radiation for the same fine sand. Jackson et al. (1967) examined the effect of soil moisture on survival of fungi and bacteria plus actinomycetes in Clarion soil. Air-dry soil or rewetted to 30% moisture was irradiated and incubated for 1 week. At a given dosage of irradiation, fewer organisms survived at the higher moisture content. They also

studied the resistance of bacteria plus actinomycetes to gamma radiation with depth in the Clarion profile. At a common dosage, the surviving fraction was largest in the A horizon, smaller in the B horizon, and smallest in the C horizon. They explained these differences in resistance by the content of soil organic matter, which may have protected some of the microorganisms from greater radiation damage.

Microbial Recolonization of Irradiated Soils

The ability of microorganisms to colonize soil sterilized by radiation without toxic effects is of importance in its application to many aspects of soil research (McLaren, 1969).

Skyring and Thompson (1966) showed that Pseudomonas denitrificans readily recolonized soil irradiated with 2.5 Mrad. Peterson (1962) demonstrated that Arthrobacter sp., Bacillus megaterium, Pesudomonas sp., Xanthomonas vesicatoria, and Trichoderma viride grew rapidly in electron sterilized soil. Salonius et al. (1967), working with Pseudomonas fluorescens and Arthrobacter globiformis, found that these two bacteria rapidly recolonized irradiated soil. Rovira and Bowen (1969) added a soil suspension to soil irradiated with 2.5 Mrad and found good colonization of heterotrophic bacteria and fungi, although nitrifying bacteria showed little activity during the first 11 weeks. On the other hand, Cawse (1975) found that pure cultures of Nitrosomonas europaea and Nitrobacter winogradski successfully recolonized in organic,

calcareous clay loam which previously had been irradiated with 8 Mrad. He also found when the perfused soil was mixed with gamma sterilized soil, nitrification was well-established within 20 days and that no nitrite accumulated.

MATERIALS AND METHODS

Rhizobium japonicum Cultures

Four strains of *Rhizobium japonicum* were used in this study. Two Beltsville strains, 110 and 123, were obtained from Dr. B. E. Caldwell (Crops Research Division, ARS, USDA, Beltsville, Maryland) and two Australian strains, CC709 and CB1809, were obtained from Dr. J. M. Vincent (102 Northwood Road, Northwood, New South Wales, 2066, Australia).

Rhizobium japonicum Culture Medium

"Medium 79" as described by Wright (1925) was used with modifications as indicated by Danso and Alexander (1974). Composition of the medium was: 10.0 g mannitol, 1.0 g yeast extract, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, and 0.1 g NaCl in 1.0 liter of distilled water. For plate counts and slant cultures, 15.0 g agar were added to each 1.0 liter of broth medium.

Difco-Bacto-Agar and Difco Bacto-Yeast Extract (Difco-Laboratories, Inc., Detroit, Michigan) were used in all experiments requiring these materials.

Selective Media for Counting Rhizobia

In obtaining a selective medium for counting rhizobia, known amounts of sterilized antibiotic were added to known volume of yeast extract mannitol agar (YEM) to give a final

desired concentration. The medium was cooled to 50°C before adding the test antibiotic and then was poured in sterilized petri dishes. After solidification, the R. japonicum strains suspended in 0.10 M phosphate buffer were transferred to the surface with a loop. Growth after 7 days was recorded following incubation at 27°C. In each test series, control plates (without antibiotics) were incubated to indicate normal growth of these strains. Strains appearing were further tested in YEM containing higher concentrations of the antibiotics by the same technique. Five replicates of each treatment were used.

Antibiotics used in this test were actidione, kanamycin, spectinomycin, and streptomycin. They were obtained from U.S. Biochem. Corp., Cleveland, Ohio.

Soils

The soils used in this study were surface samples (0-30 cm) of three Iowa soils: Hayden, Nicollet, and Okoboji. Samples were collected during the summer of 1977, air-dried at room temperature for 5 days, sieved through a 2-mm sieve, and stored in plastic bags prior to use.

Soil Analyses

Some chemical and physical properties of these soils are shown in Table 1. Soil pH and textural analysis determination were conducted on <2mm air-dry soils, total carbon and total nitrogen analysis were performed on <100 mesh air-dry soil.

Table 1. Properties of soils used to study the survival of different *R. japonicum* strains

Soil	pH	Total C	Total N	Sand	Silt	Clay
		----------%				
Hayden	6.9	3.18	0.219	54	36	10
Nicollet	6.3	3.02	0.248	43	34	23
Okoboji	6.4	6.11	0.562	18	41	41

The pH values were determined with a glass electrode (soil: water ratio, 1:2.5). Percent sand, silt, and clay were determined by the pipette method as outlined by Kilmer and Alexander (1949). Total carbon was determined by the Leco method as described by Tabatabai and Bremner (1970). Total nitrogen was determined by the method described by Bremner (1960). Soil water tensions were measured by using pressure plates. Electrical conductivity (EC) was measured according to the saturation extract procedure described by Bower and Wilcox (1965).

Sterilization

Soybean seeds were surface sterilized by immersing seeds in 75% ethyl alcohol for 3 minutes followed by vigorous agitation in a 1:1000 concentration of $HgCl_2$ solution for 3 minutes. Seeds were then rinsed several times with sterile distilled water.

Stock solutions of antibiotics were prepared in distilled

water, sterilized by filtration through a 0.45 µm Millipore filter, and stored at 4°C for up to several weeks without losing their activity. Activity was checked by control plate counts of the various strains.

Soils were sterilized by gamma radiation. Two hundred grams of air-dry soil were placed in glass containers with plastic screw caps and positioned in the core of a cobalt-60 source (Nuclear Reactor Laboratory, Ames, Iowa). Exposure was 4.2×10^6 rad for 9 hours period.

Sand, vermiculite, and cultural media were sterilized for 30 minutes in an autoclave at 121°C.

Water Activity Measurements

Soil

The relative humidities of soil at different moisture contents and salt concentrations were measured by a hygrometer instrument (American Instrument Company, Division of Travenol Laboratories, Inc., Silver Spring, Maryland 20910, USA). One hundred grams of soil were placed in a 300-cc glass container and closed. The screw-cap lid had a hole in the center just large enough to accommodate the sensor adaptor. After the soil was added to the container, the cap was quickly screwed. Relative humidities of soil at field capacity were measured by using the gray sensor and with soil at air-dry moisture were measured by using the green sensor (color of the sensor depends on its sensitivity range of measuring). The readings

of the sensor were continued until they approached equilibrium (8 hours). Duplicate readings were made for each treatment.

Growth medium

The water activity (A_w) of yeast mannitol broth (YMB) was shown to be approximately 0.999 (Bushby and Marshall, 1977a). Lower water activities in this study were obtained by the addition of glycerol to YMB. Glycerol was used instead of salts to eliminate the specific ion effect and also because glycerol has several inert properties (Dr. H. W. Walker, Department of Food Technology, Iowa State University, personal communication). Amount of glycerol needed to attain the desired A_w was calculated by using Raoult's law of mole fractions (Bone, 1973).

$$A_w = \frac{\text{Moles of water}}{\text{Moles of water + moles of solutes}}$$

Media were made by adding the appropriate quantities of glycerol to aliquots of basal medium containing a known weight of water. After the glycerol was dissolved by mechanical stirring for 15 minutes, the mixture was sterilized by autoclaving. Following autoclaving, weight loss was restored with sterile distilled water.

Soil Factors Affecting the Survival of *R. japonicum* Strains

Soil inoculum was collected by centrifugation of 4-day old rhizobia cultures grown on YEB. Cells were washed three times

with 0.1 M phosphate buffer (pH 7.1). Cell suspensions were adjusted to approximately equal turbidity for all strains. One ml of each strain was added to 30 g of gamma sterilized soil in a 60-cc glass bottle. The screw caps were not air tight allowing for gaseous exchange. Each soil was treated with three levels of NaCl (0, 0.3, and 0.7% on a dry-weight basis). The appropriate quantity of NaCl was dissolved in sterile distilled water and added in this form.

Soils were moistened to two moisture tensions. For the 0.3 bar moisture level, the desired amount of sterile distilled water and/or inoculum or salt solution was added via pipette while the soil was being thoroughly mixed in a sterilized dish. Inoculated soils were transferred to sterilized containers and incubated overnight at room temperature before estimating the number of rhizobia. Moisture contents were checked every 2 days by using 10 random samples and were periodically adjusted with sterile distilled water. Moisture contents did not vary more than $\pm 2\%$ during the incubation period. In the air-dry treatment, the desired amount of inoculum and/or salt solution was added as above. Inoculated soils were dried overnight in a cleaned air hood at room temperature before taking initial counts. Soils were then transferred to sterilized containers and incubated in desiccators over $CaCl_2$. All soils were incubated at both 28 and $36^{o}C$.

Viable counts were determined weekly by transferring 2 g soil to test tubes containing 18 ml of sterilized salts solu-

tion (a workshop conducted by J. M. Vincent, visiting professor, Oregon State University, Corvallis, Oregon, July 1976). Tubes were shaken for 1 minute on a super-mixer (Matheson Scientific). Suitable soil dilutions were plated in duplicate on YEM agar supplemented with antibiotics and the plates were incubated at 28°C for 7 days. Results were expressed as numbers of survivors per g soil.

For statistical analysis of the data, all counts were converted to logarithms. Plates containing less than 10 rhizobia per plate were given a logarithmic value of 1.0 for the purpose of calculations (Vincent et al., 1962).

Survival of R. japonicum Strains During Desiccation

Sand culture

To test the ability of R. japonicum strains to withstand desiccation, three-day-old cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml YEB, or in YEB adjusted to the minimum A_w permitting growth of rhizobia. Minimum A_w was determined by growth in varying rates of glycerol. The cultures were incubated at 28°C on a rotary shaker at 140 rpm. The cells were harvested by centrifugation after 4 days for rhizobia grown in basal medium and after 10 days for rhizobia grown in basal medium with low A_w. The cells were washed three times with 0.1 M phosphate buffer (pH 7.1) or with buffer adjusted with glycerol to the same low A_w as the basal medium. One ml of this cell suspension was added uniformly over 6.0 g

of sterilized sand contained in a 35-mm diameter petri dish. The inoculated dishes were then dried in a clean air hood at room temperature for 2-3 hours prior to storage. After drying, the viable cells were counted by the dilution technique for each strain and the numbers were considered as a zero time count. Ten plates for each strain were maintained at 28°C in a desiccator over $CaCl_2$. Dishes were withdrawn from the desiccator at regular intervals, and the numbers of survivors were counted by the dilution technique on YEM agar. Triplicate plates were poured for each sample, and the plates were incubated at 28°C for 7 days.

Broth culture

Rhizobium japonicum strains were added to YEB previously adjusted to varying water activities. One ml of culture from each strain was added in duplicate to 50 ml of broth in a 300-cc Wheaton nephelo culture flask. Flasks were continually shaken on a rotary shaker at 28°C. The daily changes in transmittance at 525 nm due to bacterial growth were measured using a Bauch & Lomb spectronic 20 colorimeter.

Water Sorption Isotherms of *R. japonicum* Strains

Apparatus

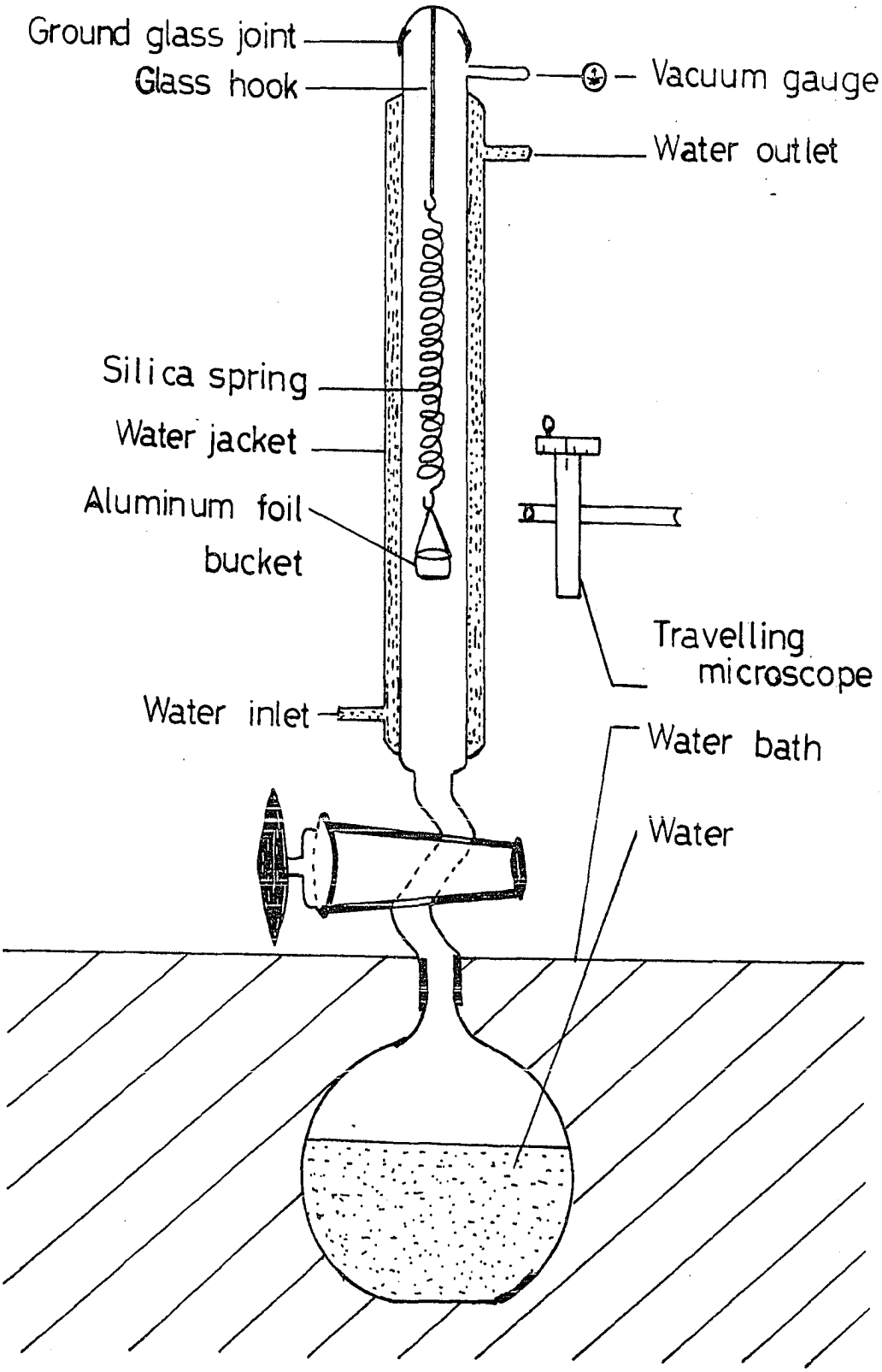
A silica spring balance as described by McBain and Baker (1926) was used to measure equilibration of rhizobial cells with water vapor. A vacuum apparatus was employed to obtain

rhizobial sorption isotherms similar to the one used by Bateman et al. (1962). The schematic diagram of this apparatus is presented in Figure 1. The unit consisted of a glass sorption chamber in which a sensitive silica spring was hung in a glass hook. Freeze-dried rhizobial cells were placed in an aluminum foil bucket attached to the lower end of the spring. A water jacket controlled to 40 $\pm$ 0.01^{o}C by a circulating water bath surrounded the sorption chamber and provided a constant saturation vapor pressure, P_o. Vapor pressure of water, P, within the chamber was determined by the temperature of water in the container immersed in a constant temperature water bath at the bottom of apparatus. This was controlled within $\pm$ 0.05^{o}C. The large stop-cock at the bottom of the chamber controlled the vapor pressure in the water container and separated the sorption chamber, during evacuation, from the saturated vapor pressure in the water container at the bottom of the chamber. Changes in the weight of the sample were measured by observing the extension of the silica spring using a travelling microscope.

Sample preparation

Rhizobium japonicum strains were grown on YEB for 6 days. In order to obtain salt-free suspensions of rhizobia, one ml of Triton X-100 was added to 100 ml of rhizobial growth culture (Dr. F. D. Williams, Bacteriology Department, Iowa State University, personal communication). Suspensions were dialyzed

Figure 1. Schematic diagram of water adsorption apparatus used to determine water adsorption isotherms for *R. japonicum* strains


Ground glass joint
Glass hook
Vacuum gauge
Water outlet
Silica spring
Water jacket
Aluminum foil bucket
Travelling microscope
Water inlet
Water bath
Water

thoroughly at 2°C for 4 days with daily replacing of the water. Treatments without Triton X-100 addition and without dialyzation were also performed. Rhizobial suspensions were frozen by using dry ice and acetone, then dried overnight in a lypholyzer. Freeze-dried samples were transferred to preweighed aluminum foil buckets and stored in a vacuum desiccators over $CaCl_2$ until used.

Procedure

Water adsorption isotherms of freeze-dried rhizobia were performed at 40°C. Bacterial samples contained in aluminum foil buckets were transferred from the vacuum desiccator and hung by the silica spring in the sorption chamber. The stopcock was closed and the sorption chamber evacuated to a pressure of 10^{-3}-10^{-4} torr (mm Hg) using a pressure control cell backed by a mechanical pump (Figure 2). After overnight evacuation, the vacuum line was closed and the weight of the sample was recorded by the magnitude of the spring extension using the travelling microscope.

Different relative vapor pressures or water activities in the sorption chamber were obtained as follows:

The relative vapor pressure or water activity = $\frac{P}{P_o}$

where

P is the vapor pressure above the sample determined by temperature of the water in the container at the bottom of the apparatus.

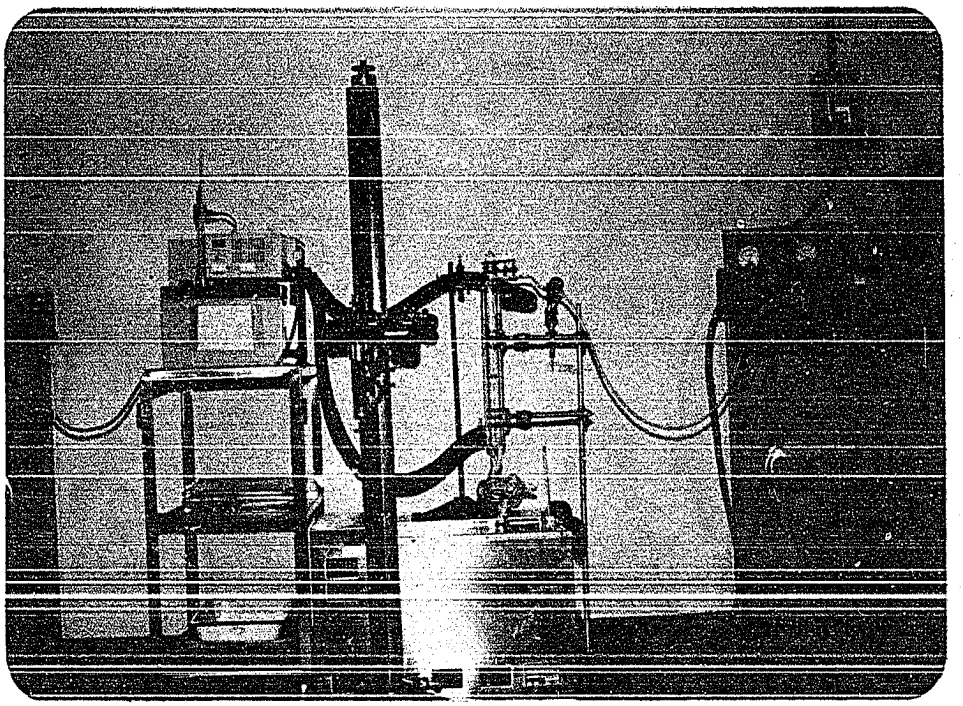


Figure 2. Water adsorption apparatus used to determine water adsorption isotherms for R. japonicum strains

P_o is the vapor pressure of water at the temperature of the jacket surrounding the sorption chamber.

From the Handbook of Chemistry and Physics (1973-1974), vapor pressure of water can be obtained at any temperature. Twelve different temperatures, from 3 to 40°C, were used to give 12 different vapor pressures (Table 27, Appendix).

To attain the desired relative humidity in the sorption chamber, the stop-cock was opened allowing the vapor in the water container to equilibrate with the evacuated sorption chamber.

Water sorption isotherms of solid egg albumin were measured to test the performance of the apparatus and the values obtained were closely related to those obtained by Bull (1944). Data of these two measurements are shown in Table 28, Appendix.

Rhizobial dimensions were measured by phase contrast microscopy with calibrated micrometer and surface areas were calculated by assuming the rhizobia to be cylinders with flat ends.

B.E.T. theory (Brunauer, Emmett, and Teller, 1938) was applied to the data from the water sorption isotherms of R. japonicum strains to calculate the active surface area.

The change in surface free energy when rhizobial cells adsorb water vapor has been calculated by application of Bangham's (1937) free energy equation to isotherm data.

Nitrogen Fixation

Nitrogen fixation was determined by the method described by Wacek and Brill (1976).

Statistical Analysis

Statistical evaluations were conducted by using the Statistical Analysis System of Service, analysis of variance and general linear model (1972 and 1976), and the Iowa State University Computation Center IBM 360 computer.

RESULTS AND DISCUSSION

Soil Characteristics Affecting the Availability of Soil Water to Microbes

Soils were selected to obtain a range in texture and organic matter content (Table 1). Water activities of Hayden sandy loam, Nicollet loam, and Okoboji silty clay were measured at two different moisture tensions (field capacity and air-dry) and at three different salinities.

Results in Table 2 show that water contents at field capacity (0.3 bar) were different for the three soils. This, as expected, was presumably due to the differences in texture and organic matter content. However, the water activity (A_w) values were 0.991, 0.990, and 0.990 for Hayden, Nicollet, and Okoboji soils, respectively. Thus, the A_w values were similar for all three soils when they were at tensions of 0.3 bar. Similar findings were observed by Bouyoucos and Cook (1967). Furthermore, they found that the addition of 1 g of water to 50 g of oven-dry soil produced tremendously different values of relative humidity in the various textured soils; in fine sand, it was 99.7%, while in Bryce silty clay the relative humidity was only 10%. Water activities of the soils were also measured under air-dry conditions. Results again indicate that similar A_w values were obtained at widely different moisture contents (Table 2).

In saline soils, not only the texture and organic matter

Table 2. Water activities and moisture contents of three different soil textures at two moisture tensions[a]

Soil	Texture	Field capacity		Air-dry	
		Moisture	A_w	Moisture	A_w
		%		%	
Hayden	Sandy loam	16.7	0.991	2.8	0.505
Nicollet	Loam	30.4	0.990	10.1	0.495
Okoboji	Silty clay	50.4	0.990	19.2	0.500

[a]Average of two replicates.

affect water availability to microorganisms but salts, in addition to their specific ion effects, are important as well. To evaluate this effect, the soils were treated with three levels of sodium chloride. Data of Table 3 show the electrical conductivity in the soil extract and the change in soil water activity with addition of different levels of salt at field capacity and at air-dry moisture contents. In all three soils, A_w values decreased by increasing salt additions.

Added salt decreased the A_w measurements more drastically in dry soils than in moist soils. The reduction in A_w due to the addition of 0.7% NaCl at field capacity was from 0.991 to 0.975, 0.990 to 0.175, and 0.990 to 0.975 in Hayden, Nicollet, and Okoboji, respectively. By contrast, the corresponding reductions were from 0.505 to 0.440, 0.495 to 0.425, and 0.500 to 0.430 for the air-dry samples. Results of this study verify

Table 3. Effects of salt addition on soil water activities at different soil moisture contents[a]

Soil	NaCl (%)	E.C. (mmhos/cm)	Water activity (A_w) Field capacity	Air-dry
Hayden	0.0	0.23	0.991	0.505
	0.3	6.30	0.982	0.465
	0.7	15.20	0.975	0.440
Nicollet	0.0	0.30	0.990	0.495
	0.3	5.80	0.980	0.465
	0.7	12.80	0.975	0.425
Okoboji	0.0	0.35	0.990	0.500
	0.3	5.15	0.984	0.470
	0.7	11.50	0.975	0.430

[a]Average of two replicates.

that the presence of salts in soil has a significant effect on availability of soil water to microorganisms, especially under drying conditions.

Selective Media for Counting Rhizobia

To detect the survival of mixed R. japonicum strains when applied to soils, a study was conducted to obtain a selective

medium which could identify each strain. Vincent[1] pointed out that R. japonicum strain CC709 was capable of multiplying on YEM supplemented with 500 µg of spectinomycin per ml, while R. japonicum strain CB1809 was capable of multiplying on YEM supplemented with 40 µg of streptomycin per ml. The capability of the two Beltsville strains, 110 and 123, to multiply on these media was determined, and results showed that these strains could not tolerate the presence of either antibiotic at these concentrations. YEM media supplemented with other antibiotics at different concentrations were therefore tested to detect antibiotics which they could tolerate (Table 4). Medium with 15 µg of kanamycin per ml YEM was found to be suitable for both strains 110 and CB1809, while medium with 40 µg of streptomycin was suitable to allow growth only of strain CB1809. Therefore, the count of strain 110 could be calculated by subtracting the growth on YEM supplemented with 40 µg of streptomycin per ml from counts on medium with 15 µg of kanamycin per ml. Rhizobia counts of strain 123 was also calculated by subtraction as indicated in Table 4.

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[1] A workshop presented by J. M. Vincent, visiting professor in the Department of Microbiology at Oregon State University, July 1976.

Table 4. *Rhizobium japonicum* strains able to grow on YEM agar supplemented with antibiotics

R. japonicum strain	Antibiotic	Concentration (µg/ml YEM)
CC709	Spectinomycin	500
CB1809	Streptomycin	40
CB1809 + 110	Kanamycin	15
CB1809 + 110 + 123	Kanamycin	10

Soil Factors Affecting the Survival of *R. japonicum* Strains

The survival of the four *R. japonicum* strains as influenced by temperature, moisture, and salt levels in different soils was investigated by comparing population changes in gamma sterilized soils. Before starting the experiment, plate counts for each soil were made on YEM agar supplemented with antibiotics to be sure that soils were free of the test strains of rhizobia. During the incubation period, control treatments of each soil without inoculum were also carried out.

An analysis of variance of the results is presented in Table 5. A comparison of F values shows that soils, treatments, strains, and time of incubation played statistically significant roles in the persistence of rhizobia in soil. The treatment effects will be evaluated more fully in the following sections.

Table 5. Analysis of variance of factors affecting survival of R. japonicum strains in three different soils

Source	Degrees of freedom	Mean square	F value
Soils	2	33.4145	22.39**
Treatments (TRT)	11	294.4793	197.33**
Error (a)	22	1.4923	
Strains (ST)	3	48.4880	212.90**
Strains x TRT	33	1.9841	8.71**
Error (b)	66	0.2277	
Time (E)	7	198.8162	340.99**
E_L (linear)	(1)	1274.6298	774.66**
E_Q (quadratic)	(1)	103.1205	62.67**
E x TRT	77	14.8610	25.49**
E x ST	21	3.7740	6.47**
E x TRT x ST	231	0.9593	1.65**
Error (c)	672		

**Significant at the 0.001 level.

Effect of salt

Table 6 indicates the main effects of moisture, temperature, and salt were highly significant at the 0.001 level. The influences of salt on the survivability of R. japonicum strains under different incubation conditions are presented in Tables 7 to 18. Generally, data revealed that salt effects

Table 6. Analysis of variance of soil treatments affecting survival of R. japonicum strains in the soil

Source	Degrees of freedom	Mean square	F value
Treatments	11	294.4793	197.33**
Moisture (M)	1	2913.5981	1952.42**
Temperature (T)	1	70.9323	47.53**
Salt (S)	2	106.6261	60.81**
S_L (linear)	(1)	213.2325	142.89**
S_Q (quadratic)	(1)	0.0197	0.01
M x T	1	22.8039	15.28**
M x S	2	8.4589	5.67*
T x S	2	0.3952	0.26
M x T x S	2	0.4883	0.33
Error (a)	22	1.4923	

**Significant at the 0.001 level.

*Significant at the 0.05 level.

varied by soils, by strains, and by soil moisture contents.

Results in Tables 7, 8, and 9 show the survival of R. japonicum strain 110 in Hayden, Nicollet, and Okoboji soils, respectively. After 7 weeks of incubation in Hayden sandy loam at the 0.3 bar moisture level, rhizobium numbers decreased by slightly greater than 1 log unit with the 0.7% addition of salt. This was the soil which had only 16.7% moisture at an

Table 7. Effects of salt levels (w/w %) on the survival of R. japonicum strain 110 in Hayden sandy loam incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.57	6.62	6.79	6.82	6.80	6.79	6.78	6.77
		0.3	6.57	6.48	6.45	6.49	6.47	6.44	6.43	6.42
		0.7	6.55	6.34	6.33	6.23	6.11	5.90	5.83	5.74
	36	0.0	6.60	6.61	6.67	6.69	6.72	6.67	6.61	6.55
		0.3	6.50	6.38	6.25	6.48	6.25	6.24	6.19	6.18
		0.7	6.56	6.54	6.50	6.41	6.06	5.83	5.50	5.32
Dry condition	27	0.0	5.81	5.61	5.50	5.24	5.29	5.18	5.08	4.98
		0.3	5.81	5.57	5.38	5.24	4.95	4.61	-[a]	4.19
		0.7	5.76	5.48	5.36	5.11	4.83	-	-	-
	36	0.0	5.79	5.53	5.34	4.98	4.77	4.52	4.03	-
		0.3	5.78	5.54	5.31	4.92	4.69	4.39	-	-
		0.7	5.74	5.42	5.01	4.76	4.13	-	-	-

[a] -, too few to be detected by procedures used.

Table 8. Effects of salt levels (w/w %) on the survival of R. japonicum strain 110 in Nicollet soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.64	6.76	6.85	6.84	6.83	6.81	6.83	6.78
		0.3	6.63	6.59	6.60	6.62	6.61	6.60	6.64	6.59
		0.7	6.65	6.50	6.45	6.52	6.33	6.31	6.28	6.15
	36	0.0	6.67	6.75	6.77	6.78	6.79	6.45	6.75	6.71
		0.3	6.63	6.63	6.68	6.61	6.52	6.45	6.37	6.27
		0.7	6.63	6.61	6.61	6.56	6.48	6.47	6.27	5.99
Dry condition	27	0.0	5.76	5.54	5.39	5.29	5.28	5.10	5.13	5.11
		0.3	5.76	5.50	5.33	5.28	5.19	4.97	4.66	-[a]
		0.7	5.76	5.50	5.38	5.18	5.10	4.87	-	-
	36	0.0	5.77	5.58	5.38	5.13	4.98	4.65	4.65	4.02
		0.3	5.76	5.55	5.34	4.98	4.79	4.59	-	-
		0.7	5.76	5.45	5.02	4.89	4.28	-	-	-

[a] -, too few to be detected by procedures used.

Table 9. Effects of salt levels (w/w %) on the survival of R. japonicum strain 110 in Okoboji soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.72	6.76	6.81	6.85	6.84	6.77	6.82	6.82
		0.3	6.69	6.67	6.65	6.65	6.63	6.61	6.61	6.60
		0.7	6.68	6.65	6.64	6.64	6.63	6.58	6.45	6.34
	36	0.0	6.67	6.62	6.69	6.71	6.68	6.66	6.67	6.62
		0.3	6.70	6.60	6.57	6.60	6.50	6.45	6.42	6.42
		0.7	6.71	6.61	6.53	6.51	6.42	6.29	6.11	5.97
Dry condition	27	0.0	5.76	5.59	5.49	5.41	5.34	5.22	5.27	5.18
		0.3	5.73	5.56	5.37	5.29	5.24	5.02	4.76	4.39
		0.7	5.73	5.51	5.54	5.20	5.06	4.91	4.65	-
	36	0.0	5.77	5.76	5.4o	5.23	501	5.08	4.87	4.50
		0.3	5.74	5.55	5.33	4.99	4.87	4.57	4.70	-
		0.7	5.73	5.47	5.11	4.91	4.43	-	-	-

[a]-, too few to be detected by procedures used.

Table 10. Effects of salt levels (w/w %) on the survival of R. japonicum strain 123 in Hayden soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.57	6.69	6.72	6.79	6.79	6.78	6.76	6.76
		0.3	6.58	6.45	6.32	6.29	6.14	6.03	6.02	5.95
		0.7	6.52	6.31	6.29	5.99	5.72	5.63	5.49	5.39
	36	0.0	6.57	6.60	6.65	6.71	6.62	6.61	6.59	6.50
		0.3	6.56	6.50	6.31	6.27	6.06	5.98	6.02	6.00
		0.7	6.45	6.36	6.03	5.86	5.79	5.25	5.10	-[a]
Dry condition	27	0.0	5.77	5.39	5.20	5.06	4.93	4.85	-	-
		0.3	5.77	5.29	5.06	4.99	4.71	-	-	-
		0.7	5.72	5.11	4.97	4.84	3.69	-	-	-
	36	0.0	5.83	5.24	5.04	4.86	4.45	-	-	-
		0.3	5.80	5.20	4.98	4.78	4.08	-	-	-
		0.7	5.77	5.04	4.85	-	4.09	-	-	-

[a] -, too few to be detected by procedures used.

Table 11. Effects of salt levels (w/w %) on the survival of R. japonicum strain 123 in Nicollet soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.62	6.62	6.79	6.78	6.76	6.74	6.71	6.70
		0.3	6.63	6.57	6.50	6.50	6.48	6.47	6.41	6.31
		0.7	6.66	6.50	6.39	6.34	6.24	5.97	5.79	5.73
	36	0.0	6.68	6.69	6.73	6.61	6.59	6.59	6.57	6.56
		0.3	6.69	6.53	6.59	6.46	6.30	6.24	5.97	5.90
		0.7	6.69	6.46	6.37	6.23	6.00	5.84	5.72	5.47
Dry condition	27	0.0	5.80	5.42	5.27	5.11	5.00	4.89	4.81	-[a]
		0.3	5.77	5.33	5.24	5.06	4.85	4.69	-	-
		0.7	5.70	5.28	5.04	5.02	4.68	3.69	-	-
	36	0.0	5.80	5.29	5.13	4.94	4.90	4.50	-	-
		0.3	5.77	5.27	5.10	4.95	4.57	-	-	-
		0.7	5.72	5.04	4.88	4.67	-	-	-	-

[a]-, too few to be detected by procedures used.

Table 12. Effects of salt levels (w/w %) on the survival of R. japonicum strain 123 in Okoboji soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.71	6.74	6.81	6.80	6.79	6.78	6.78	6.76
		0.3	6.72	6.69	6.59	6.55	6.58	6.54	6.50	6.49
		0.7	6.69	6.57	6.52	6.28	6.03	5.99	5.93	5.82
	36	0.0	6.70	6.68	6.61	6.61	6.63	6.57	6.48	6.43
		0.3	6.68	6.55	6.49	6.44	6.25	5.98	6.04	6.02
		0.7	6.69	6.50	6.39	6.32	6.20	6.04	5.87	5.58
Dry condition	27	0.0	5.76	5.43	5.22	5.14	5.08	4.93	4.92	-[a]
		0.3	5.75	5.35	5.23	5.08	4.93	4.72	3.96	-
		0.7	5.72	5.31	5.09	5.05	4.81	4.10	-	-
	36	0.0	5.77	5.32	5.20	4.96	4.96	4.61	4.02	-
		0.3	5.74	5.29	5.13	5.00	4.70	4.00	-	-
		0.7	5.72	5.08	4.89	4.77	4.03	-	-	-

[a] -, too few to be detected by procedures used.

Table 13. Effects of salt levels (w/w %) on the survival of R. japonicum strain CC709 in Hayden soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.60	6.68	6.82	6.86	6.83	6.81	6.79	6.78
		0.3	6.58	6.57	6.49	6.51	6.54	6.59	6.57	6.55
		0.7	6.54	6.45	6.32	6.31	6.22	6.02	5.86	5.80
	36	0.0	6.51	6.62	6.68	6.75	6.76	6.73	6.68	6.62
		0.3	6.51	6.41	6.48	6.42	6.34	6.29	6.33	6.27
		0.7	6.50	6.38	6.50	6.47	6.32	6.07	5.78	5.62
Dry condition	27	0.0	5.86	5.51	5.40	5.32	5.20	5.15	5.06	5.00
		0.3	5.82	5.55	5.34	5.28	4.97	4.74	4.03	-[a]
		0.7	5.77	5.50	5.31	5.18	4.87	3.99	-	-
	36	0.0	5.76	5.46	5.30	5.04	4.88	4.60	4.17	-
		0.3	5.73	5.48	5.30	4.96	4.87	4.51	-	-
		0.7	5.73	5.11	4.81	4.39	-	-	-	-

[a] -, too few to be detected by procedures used.

Table 14. Effects of salt levels (w/w %) on the survival of R. japonicum strain CC709 in Nicollet soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.65	6.61	6.79	6.85	6.83	6.83	6.79	6.79
		0.3	6.64	6.62	6.71	6.62	6.61	6.61	6.59	6.59
		0.7	6.60	6.57	6.56	6.49	6.45	6.42	6.31	6.21
	36	0.0	6.67	6.70	6.72	6.79	6.77	6.76	6.75	6.73
		0.3	6.67	6.69	6.69	6.68	6.59	6.46	6.54	6.39
		0.7	6.66	6.62	6.63	6.53	6.48	6.45	6.39	6.13
Dry condition	27	0.0	5.82	5.62	5.41	5.39	5.27	5.16	5.19	5.10
		0.3	5.79	5.54	5.33	5.31	5.22	5.02	4.69	-[a]
		0.7	5.77	5.54	5.35	5.20	5.10	4.90	3.24	-
	36	0.0	5.80	5.54	5.39	5.16	4.97	4.81	4.67	-
		0.3	5.79	5.53	5.36	5.22	5.02	4.72	-	-
		0.7	5.77	5.47	5.18	5.08	4.85	4.05	-	-

[a] -, too few to be detected by procedures used.

Table 15. Effects of salt levels (w/w %) on the survival of R. japonicum strain CC907 in Okoboji soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.72	6.79	6.80	6.80	6.80	6.79	6.78	6.77
		0.3	6.60	6.61	6.61	6.61	6.60	6.60	6.59	6.57
		0.7	6.67	6.59	6.61	6.57	6.56	6.48	6.34	6.31
	36	0.0	6.69	6.62	6.66	6.68	6.62	6.59	6.57	6.50
		0.3	6.61	6.57	6.60	6.56	6.52	6.48	6.49	6.44
		0.7	6.59	6.51	6.49	6.50	6.46	6.33	6.25	6.07
Dry condition	27	0.0	5.81	5.62	5.51	5.46	5.36	5.27	5.24	5.22
		0.3	5.78	5.57	5.41	5.34	5.25	5.10	4.77	4.50
		0.7	5.77	5.56	5.38	5.23	5.16	5.00	4.76	3.54
	36	0.0	5.79	5.57	5.41	5.19	5.04	5.02	4.85	4.34
		0.3	5.79	5.45	5.36	5.16	5.10	4.90	4.32	-[a]
		0.7	5.76	5.48	5.19	5.11	4.92	4.15	-	-

[a] -, too few to be detected by procedures used.

Table 16. Effects of salt levels (w/w %) on the survival of R. japonicum strain CB1809 in Hayden soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.57	6.66	6.79	6.78	6.85	6.79	6.79	6.76
		0.3	6.58	6.42	6.40	6.42	6.33	6.31	6.29	6.27
		0.7	6.54	6.29	6.10	5.99	5.68	5.56	5.28	5.32
	36	0.0	6.60	6.57	6.61	6.69	6.62	6.57	6.51	6.48
		0.3	6.45	6.19	6.10	5.79	5.93	5.55	5.60	5.50
		0.7	6.44	6.16	6.11	5.60	5.76	5.39	4.75	-[a]
Dry condition	27	0.0	5.76	5.34	5.13	4.99	4.89	4.81	-	-
		0.3	5.79	5.27	5.00	4.94	4.59	-	-	-
		0.7	5.72	5.06	4.89	4.79	-	-	-	-
	36	0.0	5.77	5.23	5.02	4.81	4.39	-	-	-
		0.3	5.77	5.22	4.93	4.65	4.01	-	-	-
		0.7	5.76	4.98	4.60	3.89	-	-	-	-

[a] -, too few to be detected by procedures used.

Table 17. Effects of salt levels (w/w %) on the survival of R. japonicum strain CB1809 in Nicollet soil

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.61	6.61	6.79	6.86	6.79	6.79	6.78	6.77
		0.3	6.63	6.51	6.46	6.49	6.37	6.36	6.34	6.30
		0.7	6.63	6.48	6.47	6.39	6.35	6.19	5.98	5.94
	36	0.0	6.64	6.67	6.71	6.74	6.67	6.60	6.59	6.56
		0.3	6.65	6.47	6.32	6.44	6.18	6.05	5.87	5.76
		0.7	6.62	6.48	6.35	6.22	6.00	5.87	5.66	5.43
Dry condition	27	0.0	5.75	5.47	5.24	5.13	5.02	4.94	4.84	-[a]
		0.3	5.73	5.35	5.25	4.98	4.90	4.59	-	-
		0.7	5.72	5.19	4.94	4.83	4.57	-	-	-
	36	0.0	5.76	5.29	5.06	4.87	4.39	-	2.24	-
		0.3	5.71	5.24	4.95	4.69	4.31	-	-	-
		0.7	5.72	5.09	4.81	4.40	-	-	-	-

[a]-, too few to be detected by procedures used.

Table 18. Effects of salt levels (w/w %) on the survival of R. japonicum strain CB1809 in Okoboji soil incubated at different temperatures and moisture contents

Moisture	Temp °C	% NaCl	Survival (log count g^{-1} soil) at various weeks							
			0	1	2	3	4	5	6	7
Moist condition	27	0.0	6.71	6.76	6.76	6.77	6.76	6.75	6.75	6.74
		0.3	6.63	6.62	6.57	5.56	6.49	6.48	6.47	6.43
		0.7	6.69	6.56	6.46	6.24	5.99	5.87	5.83	5.72
	36	0.0	6.67	6.61	6.57	6.50	6.52	6.50	6.46	6.45
		0.3	6.60	6.57	6.50	6.46	6.28	6.02	5.92	5.98
		0.7	6.73	6.48	6.34	6.29	6.27	6.08	5.84	5.50
Dry condition	27	0.0	5.79	5.48	5.33	5.20	5.11	5.02	4.95	-[a]
		0.3	5.75	5.34	5.24	5.03	4.89	4.59	-	-
		0.7	5.72	5.24	5.00	4.91	4.76	4.19	-	-
	36	0.0	5.75	5.29	5.14	4.94	4.77	4.51	-	-
		0.3	5.77	5.75	5.03	4.90	4.56	3.94	-	-
		0.7	5.71	5.13	4.95	4.57	3.69	-	-	-

[a] -, too few to be detected by the procedures used.

A_w of 0.975. In Nicollet loam and Okoboji silty clay, soils with greater percent moisture at a given A_w, the numbers were found to decrease by less than 1 log unit. By contrast under dry conditions, too few organisms were detected to count in all three soils when 0.3 and 0.7% salt levels were added at 36°C. At 27°C, only the Nicollet soil had too few organisms to count at the 0.3% salt level.

The survival of R. japonicum strain 123 in Hayden, Nicollet, and Okoboji is shown in Tables 10, 11, and 12, respectively. This strain was found to have lower survival rates than strain 110 under conditions of similar environmental stress. Results after 7 weeks of incubation in Hayden sandy loam showed that the decrease in numbers was from 6.76 to 5.39 log under moist conditions at 27°C and from 6.45 to too few to count at 36°C with 0.7% added salt. By contrast with dry conditions too few numbers were detected only after 5 weeks of incubation. Better survivability of rhizobia was again found at a given level of salt in Nicollet loam and Okoboji silty clay compared with Hayden sandy loam.

Results in Tables 13, 14, and 15 represent the survival of R. japonicum strain CC709 in Hayden, Nicollet, and Okoboji soils, respectively. Numbers of rhizobia were found to decrease in a similar manner to R. japonicum strain 110. On the other hand, data for R. japonicum strain CB1809 (presented in Tables 16, 17, and 18) reveal that the behavior of CB1809 under all soil treatments was similar to strain 123.

Statistical analysis of the data showed that the viable count of rhizobia decreased linearly as the salt was increased from 0 to 0.7% (Table 6). Johnson and Guenzi (1963) studied the influence of salt on ammonium oxidation and CO_2 evolution from soil. They also found the osmotic tension reduced nitrate production and CO_2 evolution in a linear manner as the salt concentration of the soil increased. Laura (1977) found that the amount of accumulated mineral nitrogen generally decreased with increasing soil salinity.

The negative effect of salt addition on rhizobia in dry soils was greater than its effect in moist soils. This effect might be attributed to the physical binding by salt of the smaller amounts of water in the dry soils, resulting in less available water to the microorganisms. As shown in Table 3, the decrease in soil water activity due to the addition of NaCl was greater in dry soils than soils at field capacity.

Theoretically, ions in aqueous solutions can affect a biological system directly by the specific ion effect, or indirectly through their influence on the effective concentration or activity of solvent water. Brown (1964) pointed out that the separation of the effects of ions from water activity is not easy; however, he concluded that the direct ionic effect is major and that water activity exerts, at most, a minor influence on bacteria in aqueous systems. Laura (1974) suggested that the influence of added salts on ammonification might be due to their effect on the degree of dissocia-

tion of water. Wilson and Norris (1970) found that the multiplication of R. japonicum strain CB756 was not affected by a salt concentration of 40 meq NaCl/l of broth but was reduced at 80 and completely inhibited at 160 meq NaCl/l. They explained that the long lag period at 80 meq/l was due to either (1) a small population of salt tolerant cells initially present, or (2) that the strain was able to adapt with time to this level of salt.

Effects of temperature and moisture

The influence of temperature and soil moisture contents on the population density of the R. japonicum strains was evaluated by desiccating soils over $CaCl_2$ and comparing these soils with those at 0.3 bar tension (field capacity). Incubation was evaluated at both 27 and 36°C.

The moisture content of the soil played a dominant role in the survivability of all R. japonicum strains studied. A comparison of F values in Table 6 shows that temperature and salt also played statistically significant roles, but to a lesser degree than moisture.

The effects of temperature and soil moisture on the survival of R. japonicum strains 110, 123, CC709, and CB1809 are illustrated in Figures 3, 4, 5, and 6, respectively. As shown in these figures, the most marked decline in rhizobia numbers occurred when soils were desiccated and incubated at 36°C. Decline also occurred when soils were desiccated and

Figure 3. Effects of temperature and moisture, O 27°C and field capacity, ● 36°C and field capacity, Δ 27°C and dry soil, and ▲ 36°C and dry soil, on the survival of R. japonicum strain 110 in (A) Hayden, (B) Nicollet, and (C) Okoboji soils

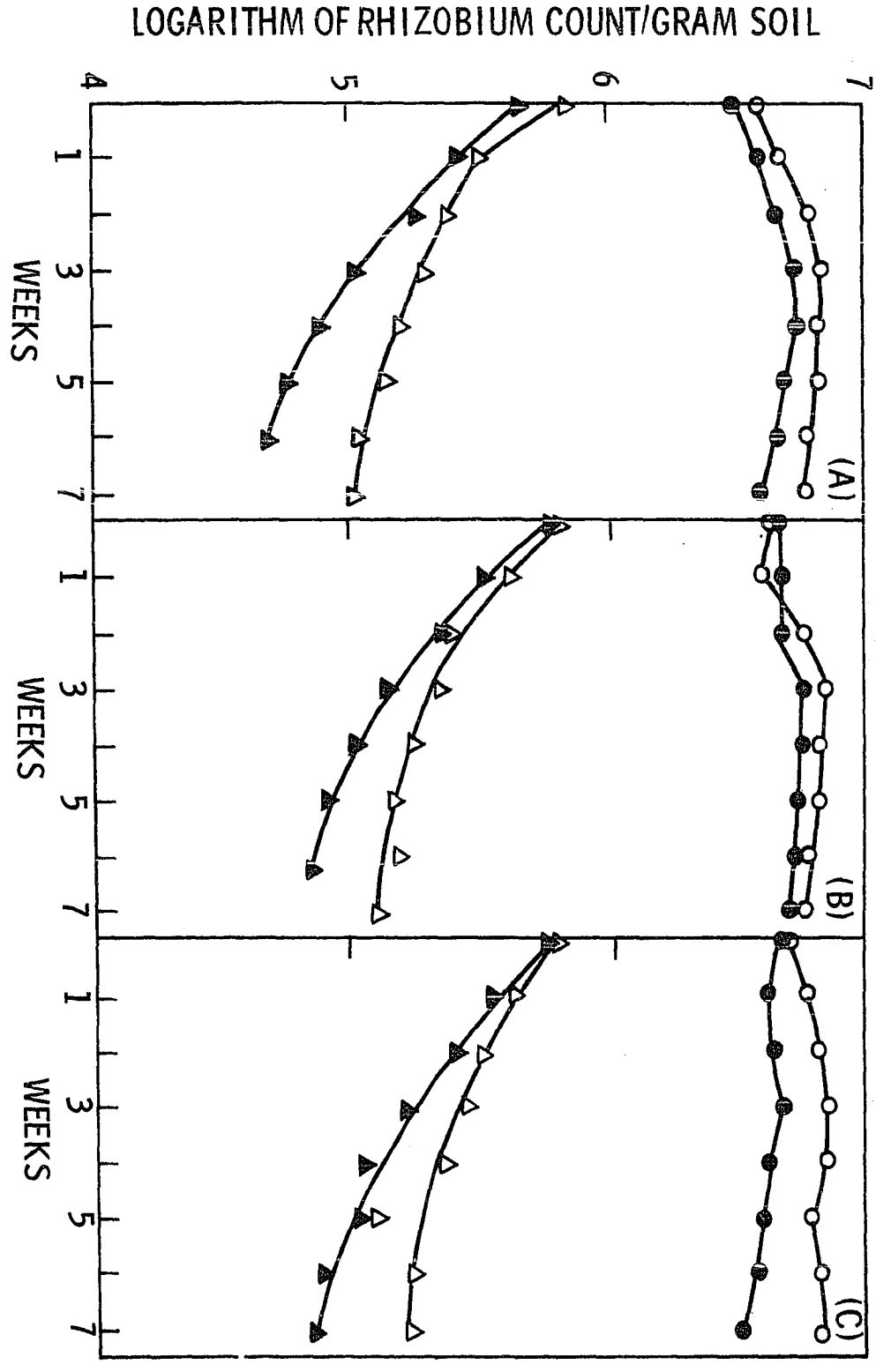

LOGARITHM OF RHIZOBIUM COUNT/GRAM SOIL
4
5
6
7
1
3
5
7
WEEKS
(A)
(B)
(C)

Figure 4. Effects of temperature and moisture, O 27°C and field capacity, ● 36°C and field capacity, Δ 27°C and dry soil, and ▲ 36° and dry soil, on the survival of R. japonicum strain 123 in (A) Hayden, (B) Nicollet, and (C) Okoboji soils

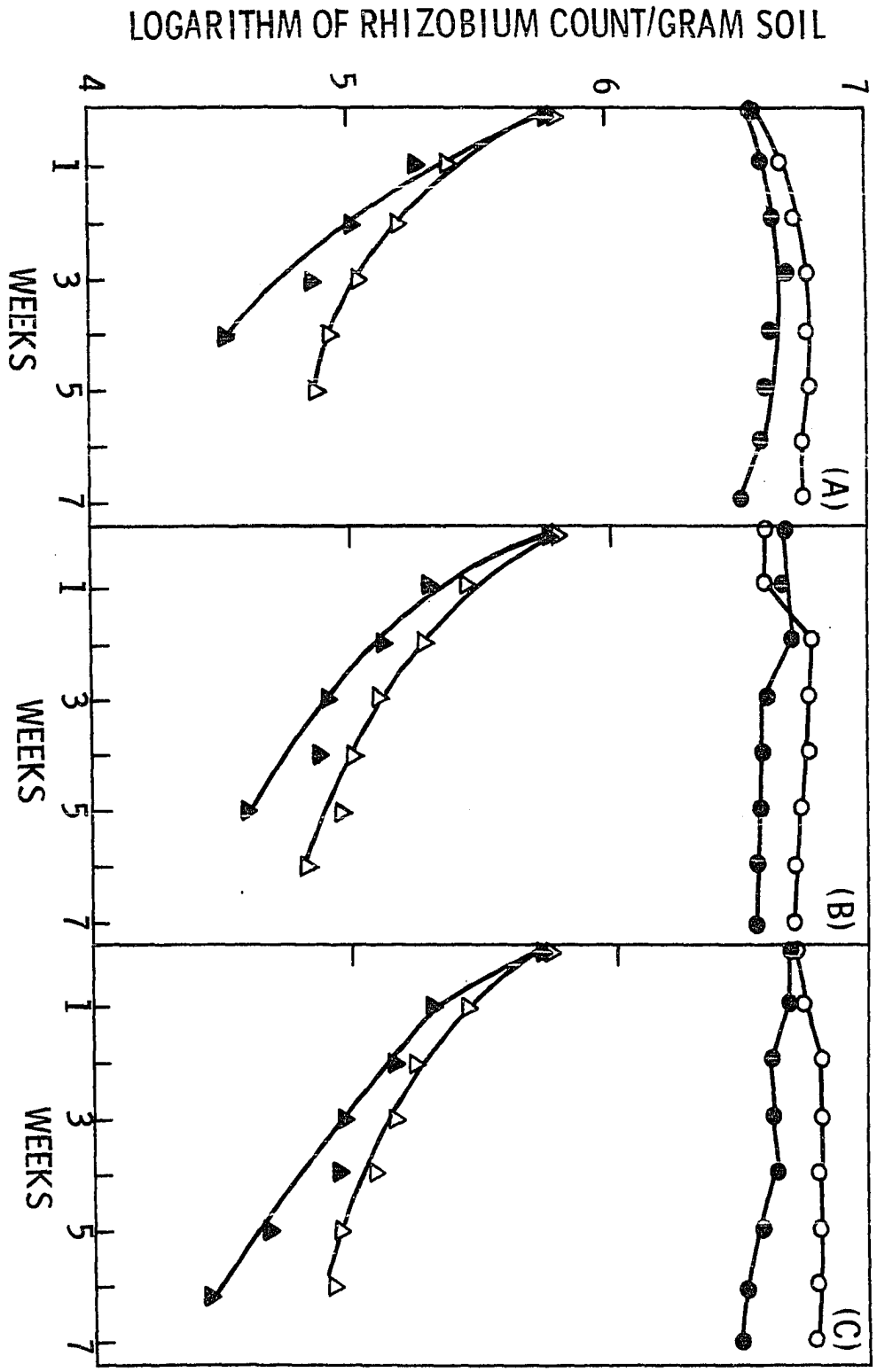

LOGARITHM OF RHIZOBIUM COUNT/GRAM SOIL
4
5
6
7
1
3
5
7
WEEKS
(A)
(B)
(C)

Figure 5. Effects of temperature and moisture, O 27°C and field capacity, ● 36°C and field capacity, Δ 27°C and dry soil, and ▲ 36°C and dry soil, on the survival of R. japonicum strain CC709 in (A) Hayden, (B) Nicollet, and (C) Okoboji soils

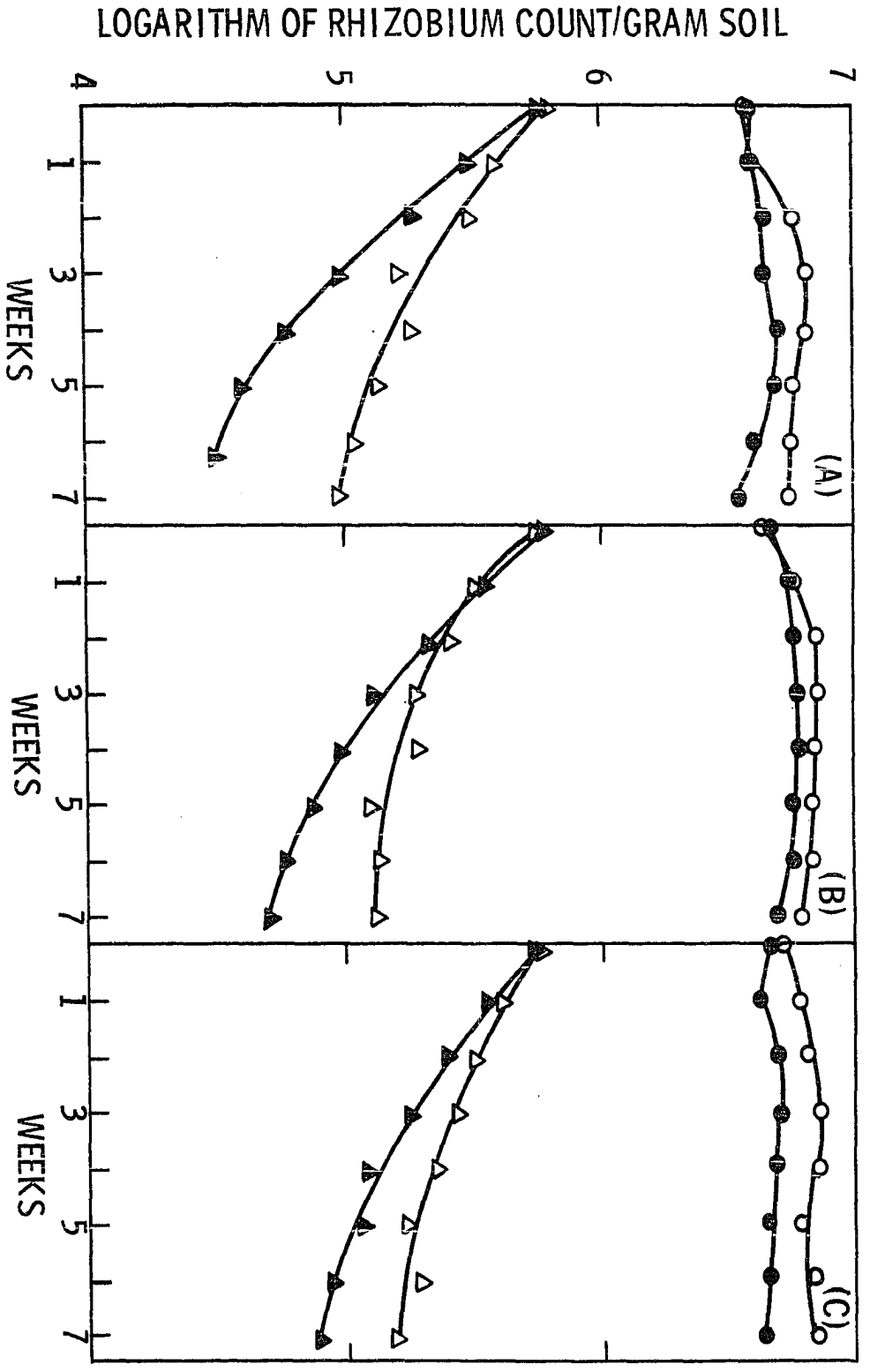
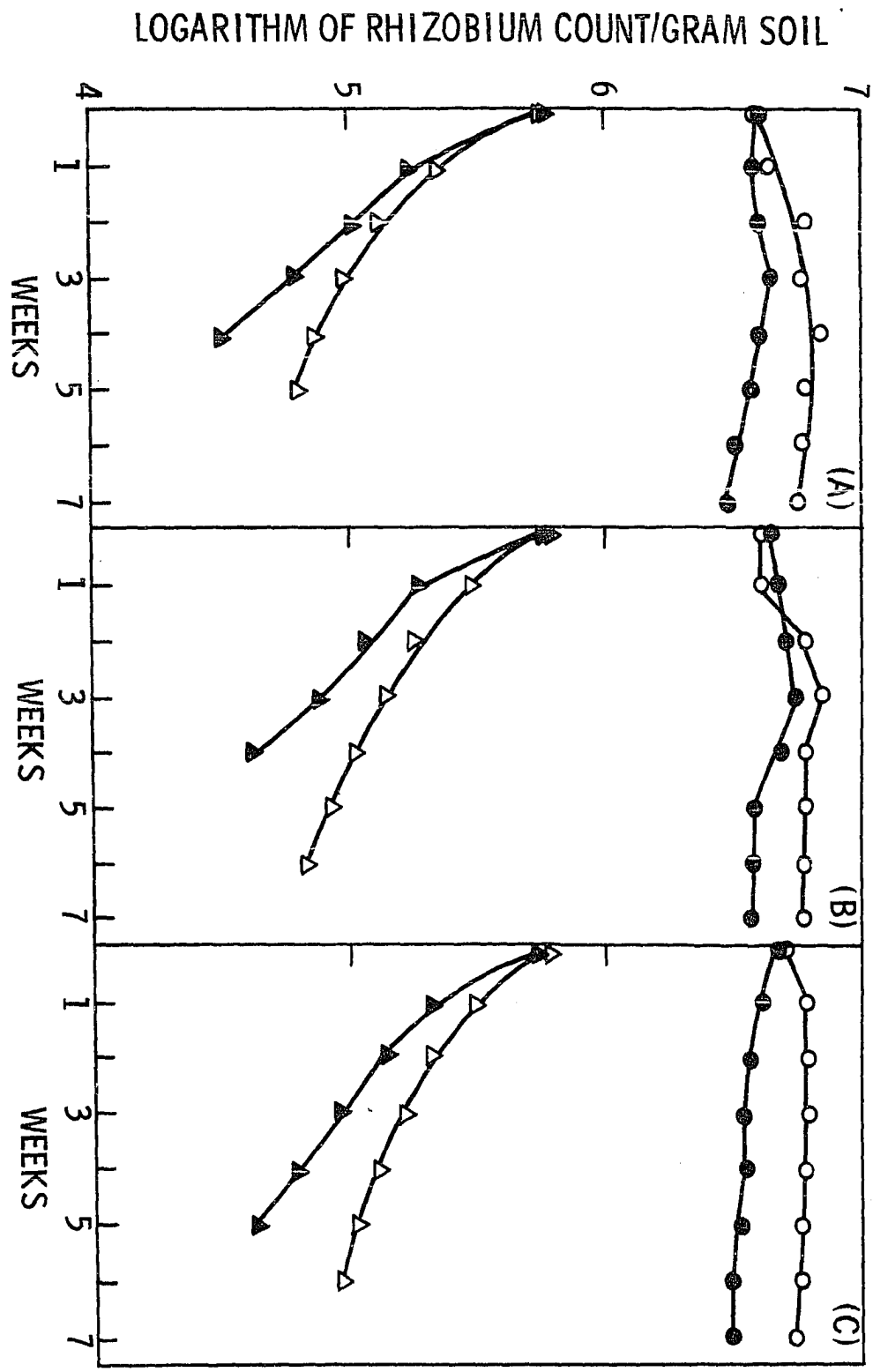

LOGARITHM OF RHIZOBIUM COUNT/GRAM SOIL
4
5
6
7
1
3
5
7
WEEKS
(A)
(B)
(C)

Figure 6. Effects of temperature and moisture, O 27°C and field capacity, ● 36°C and field capacity, Δ 27°C and dry soil, and ▲36° and dry soil, on the survival of R. japonicum strain CB1809 in (A) Hayden, (B) Nicollet, and (C) Okoboji soils


LOGARITHM OF RHIZOBIUM COUNT/GRAM SOIL
4
5
6
7
WEEKS
1
3
5
7
(A)
(B)
(C)

incubated at 27°C, but to a lesser degree than at 36°C. In soils at field capacity, the numbers of cells of all strains increased in the first few weeks after incubation; after a period, the number of cells gradually declined, but in all soils, however, the population densities present at the end of 7 weeks were higher than the numbers initially introduced. Larger decreases in rhizobia numbers occurred in soils moistened to field capacity and incubated at 36°C compared with incubation at 27°C, but high counts were still evident after 7 weeks. An analysis of variance of the strains and soil treatments affecting their survival is presented in Table 19.

Results obtained in this study provide further support for the effect of temperature and soil moisture contents on the survival of rhizobia in soils. A number of reports suggest that rhizobia persist longer at lower than at elevated temperature (Bowen and Kennedy, 1959; van Schrevan, 1970; and Danso and Alexander, 1974).

It has been reported that R. japonicum (Sen and Sen, 1956) and R. meliloti (Jensen, 1961) persist for long periods in air-dried soils, but conclusions on viability of Rhizobium sp. in these investigations were based on the presence of nodules on leguminous plants grown in such soils. It is possible that only a small number of the millions of cells introduced in the soils survived and formed nodules. Vincent (1965) indicated that temperature and loss of water were

Table 19. Analysis of variance of four R. japonicum strains and soil treatments affecting their survival in the soil

Source	Degrees of freedom	Mean square	F value
Strains (ST)	3	48.4880	212.90**
TRT x ST	33	1.9841	8.71**
M x ST	3	15.5625	68.33**
T x ST	3	0.0874	0.38
S x ST	6	0.5595	2.46*
M x T x ST	3	0.5758	2.53
M x S x ST	6	1.4864	6.53**
T x S x ST	6	1.4864	6.53**
T x S x ST	6	0.3421	1.50
M x T x S x ST	6	0.4117	1.81
Error (b)	66	0.2275	

**Significant at the 0.001 level.

*Significant at the 0.05 level.

important factors in survival of clover rhizobia. Vincent et al. (1962), working with R. trifolii applied to glass beads and seeded to subterranean clover, found under drying conditions two distinct phases of rhizobial survival: an early period in which the main loss of water occurred and in which there was rapid death, and a storage period in which the death rate was reduced. Recently, Davidson and Reuszer (1978)

observed that the survival of R. japonicum strain 61A68 on soybean seeds, with and without coating materials, at 3 weeks was from 0.9 to 19.5, 0.5 to 7.5, and 0.1 to 1.6% of the original inoculum at 15, 22.5, and 30°C, respectively. The counts of four R. japonicum strains recovered from desiccated soils at two different temperatures in the present study support the view that high temperature and loss of water were important factors in survival of soybean rhizobia. On the other hand, R. japonicum strains were found to significantly differ in their susceptibility to desiccation. As shown in Table 20, R. japonicum strain 110 did not significantly differ from strain CC709, and strain 123 did not significantly differ from strain CB1809 at the 0.01 confidence level. However, strains 110 and CC709 were significantly different from strains 123 and CB1809.

Differences between soils significantly affected survival of rhizobia as indicated in Table 5. When the soils were desiccated, less survival of the four strains was found in Hayden sandy loam than in Nicollet loam and Okoboji silty clay. Differences in soils can be attributed to the variation in clay and organic carbon contents. Marshall (1964) found that montmorillonite amendment of sandy soil protected R. trifolii from the adverse effects of drying and exposure to high temperature. Marshall (1968) also studied the relationship between the ionogenic surfaces of various rhizobia and the clay minerals. From this study, Marshall proposed that

Table 20. Mean of log counts of R. japonicum strains as affected by soil treatments

R. japonicum strain	No. of observations	Mean (log count g^{-1} soil)	L.S.D. 0.01	L.S.D. 0.05
110	576	5.520	0.075	0.056
123	576	5.090		
CC709	576	5.588		
CB1809	576	5.022		

most slower-growing rhizobia possess a simple carboxyl surface on which the clay platelets arrange themselves in a very efficient edge-to-face association; many of the faster-growing strains (e.g., R. trifolii) possess amino groups on the surface, and with a less efficient stacking of the clay particles there is less protection against desiccation.

Susceptibility of R. japonicum Strains to Desiccation

Significant differences were obtained between strains in ability to survive in desiccated soils. As shown in Figures 7, 8, and 9 for Hayden, Nicollet, and Okoboji soils, respectively, R. japonicum strains 123 and CB1809 were more sensitive to desiccation than strains 110 and CC709 in all three soils studied.

Susceptibility to desiccation of these strains was also studied in sterilized sand desiccated over $CaCl_2$. After 36

Figure 7. The survival of R. japonicum strains in Hayden sandy loam incubated at 27°C under dry conditions

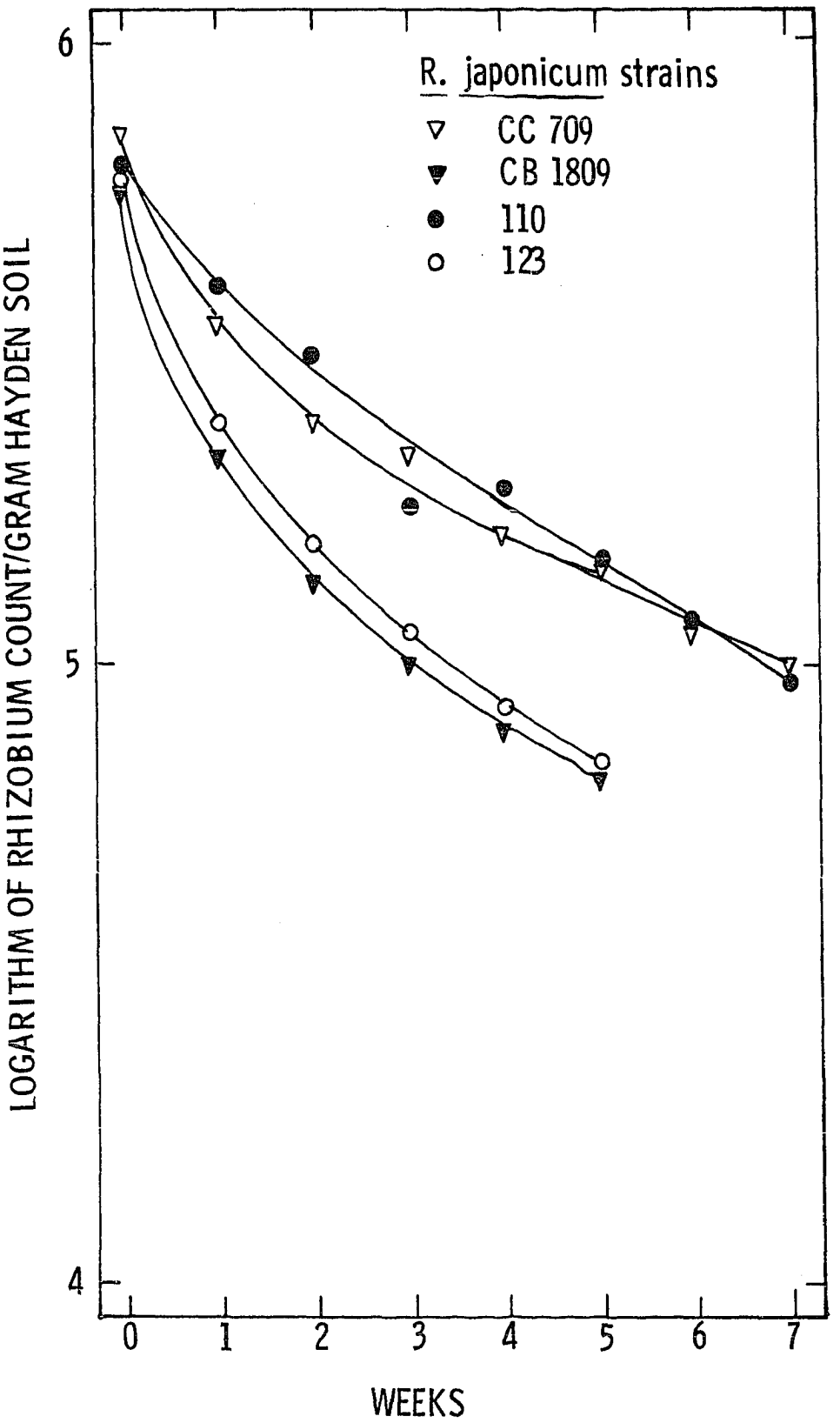

R. japonicum strains
CC 709
CB 1809
110
123
LOGARITHM OF RHIZOBIUM COUNT/GRAM HAYDEN SOIL
6
5
4
0
1
2
3
4
5
6
7
WEEKS

Figure 8. The survival of R. japonicum strains in Nicollet loam incubated at 27°C under dry conditions

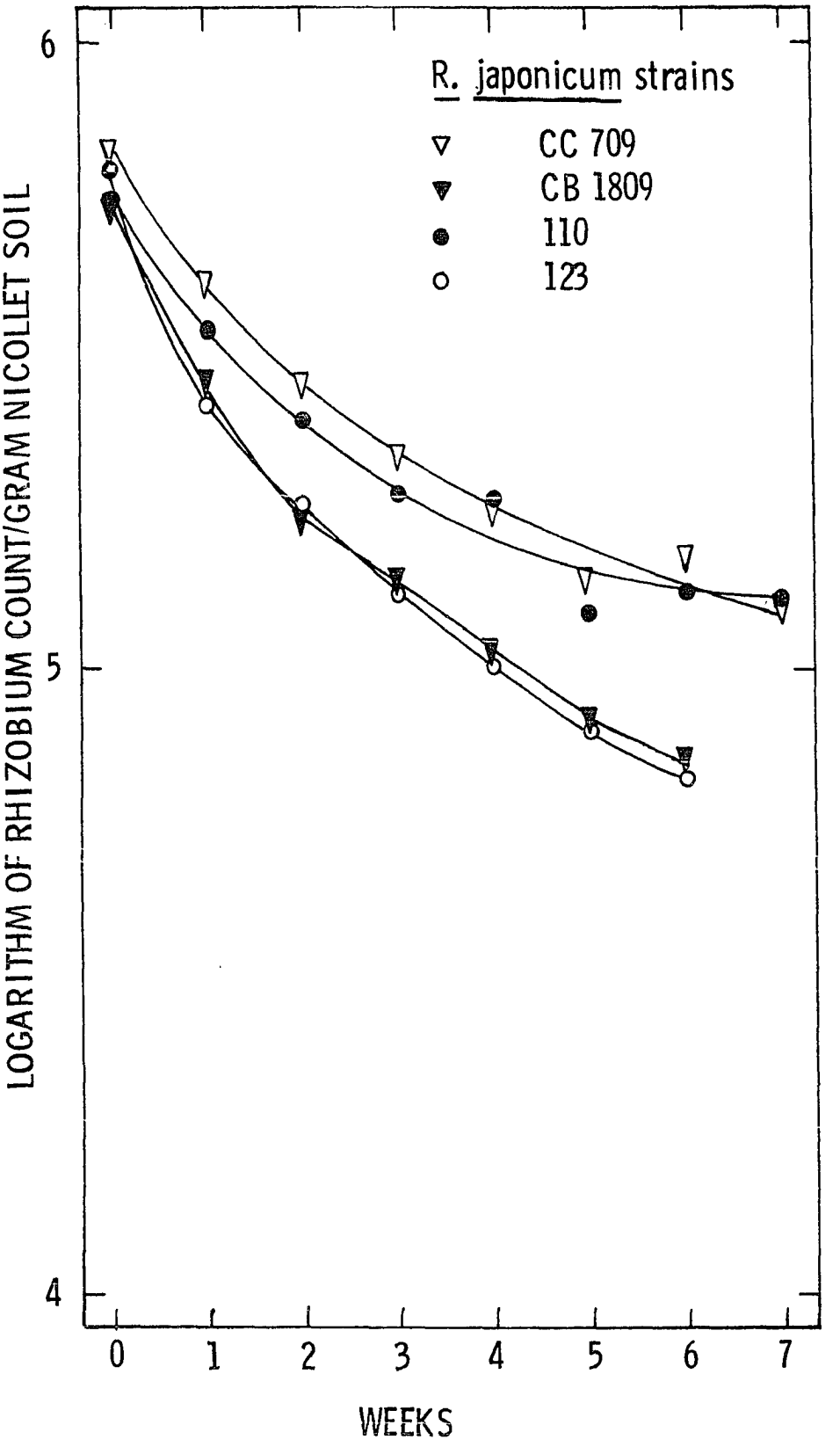
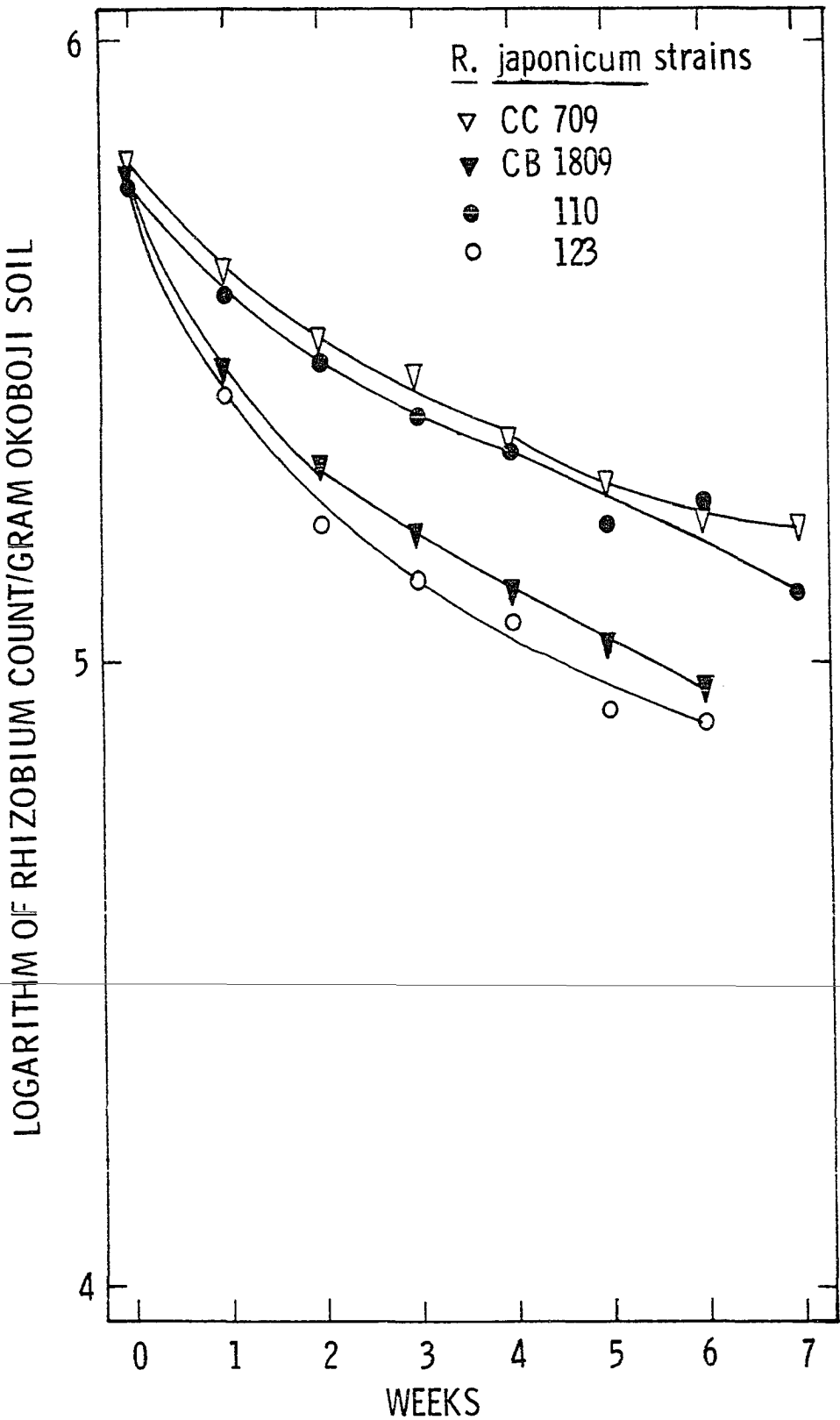

R. japonicum strains
CC 709
CB 1809
110
123
LOGARITHM OF RHIZOBIUM COUNT/GRAM NICOLLET SOIL
6
5
4
0
1
2
3
4
5
6
7
WEEKS

Figure 9. The survival of *R. japonicum* strains in Okoboji silty clay incubated at 27°C under dry conditions


R. japonicum strains
CC 709
CB 1809
110
123
LOGARITHM OF RHIZOBIUM COUNT/GRAM OKOBOJI SOIL
6
5
4
0
1
2
3
4
5
6
7
WEEKS

days, the difference in survival between strain 123 and both 110 and CC709 strains was greater than 1 log unit, and between strain CB1809 and both 110 and CC709 was approximately 2 log units (Figure 10). This is further evidence for the existence of strain differences of rhizobia due to desiccation.

Additionally, a comparison was made of the survivability of R. japonicum strains grown in YEB at an A_w value of 0.999, and in the same medium adjusted with glycerol to lower A_w values. As shown in Figure 11, lower survivability of strains 123 and CB1809 was obtained at A_w values of 0.990, 0.985, and 0.980 compared with the other two strains, 110 and CC709. The minimum A_w value allowing growth of strains 123 and CB1809 was 0.980. The growth curves of all strains at this A_w are illustrated in Figure 12. The log phase of growth of all four strains began at approximately 3 days; however, the rate of growth of 110 and CC709 was greater than that of 123 and CB1809. Thus, some mechanism allowed strains 110 and CC709 to be less adversely affected at lower A_w values.

Results obtained both in sand and broth cultures were in general agreement with results obtained in different soils for the susceptibility of the various strains to desiccation. This suggests that rapid screening of R. japonicum strains for desiccation resistance can be accomplished by growing different strains in media supplemented with glycerol to different A_w values.

Survival of many Gram-negative bacteria decreases when

Figure 10. The survival of R. japonicum strains in sterile sand incubated at 27°C

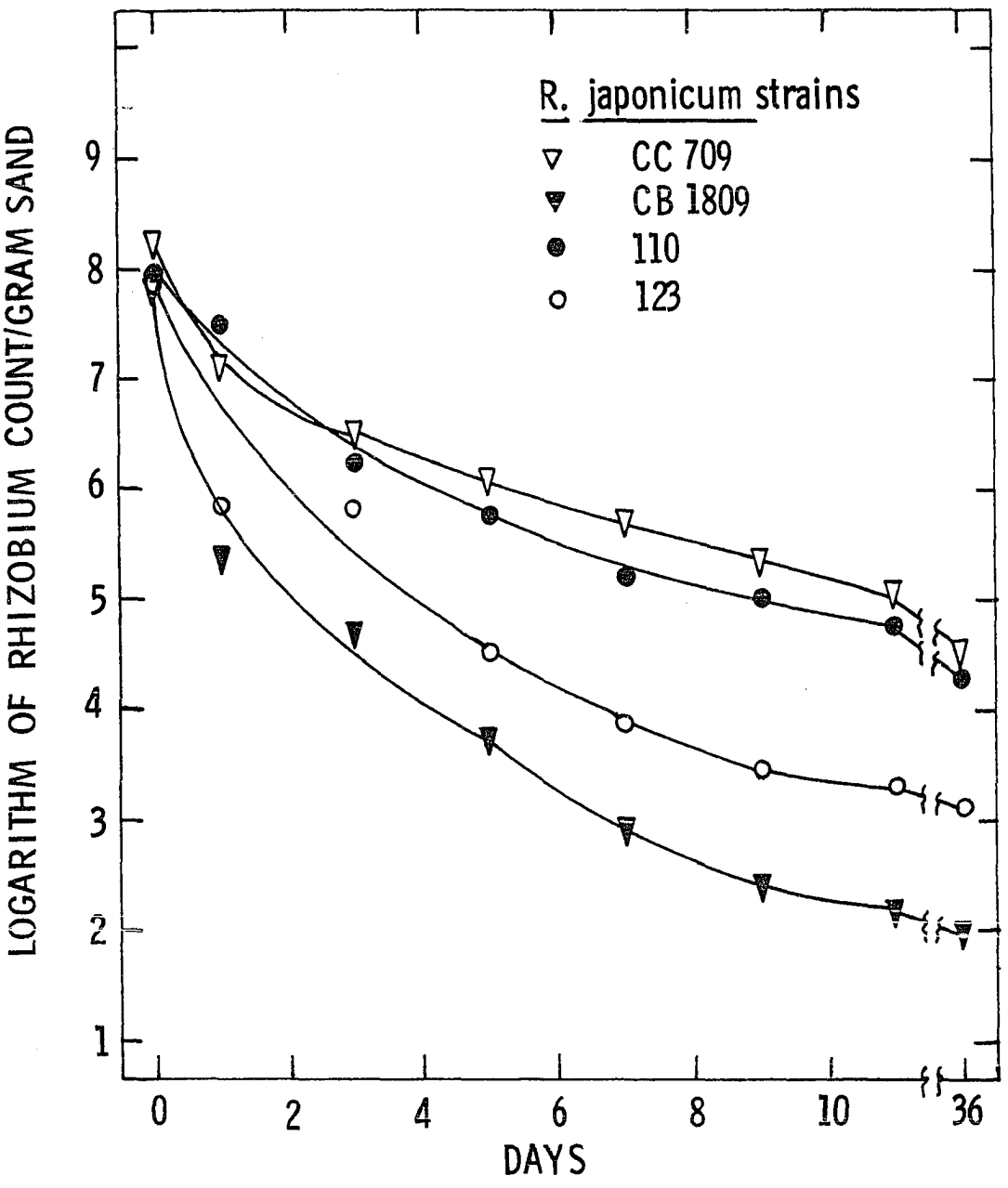

R. japonicum strains
CC 709
CB 1809
110
123
LOGARITHM OF RHIZOBIUM COUNT/GRAM SAND
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
36
DAYS

Figure 11. The effect of water activity (A_w) as adjusted by glycerol on the 8-day growth of R. japonicum strains in yeast extract broth (YEB)

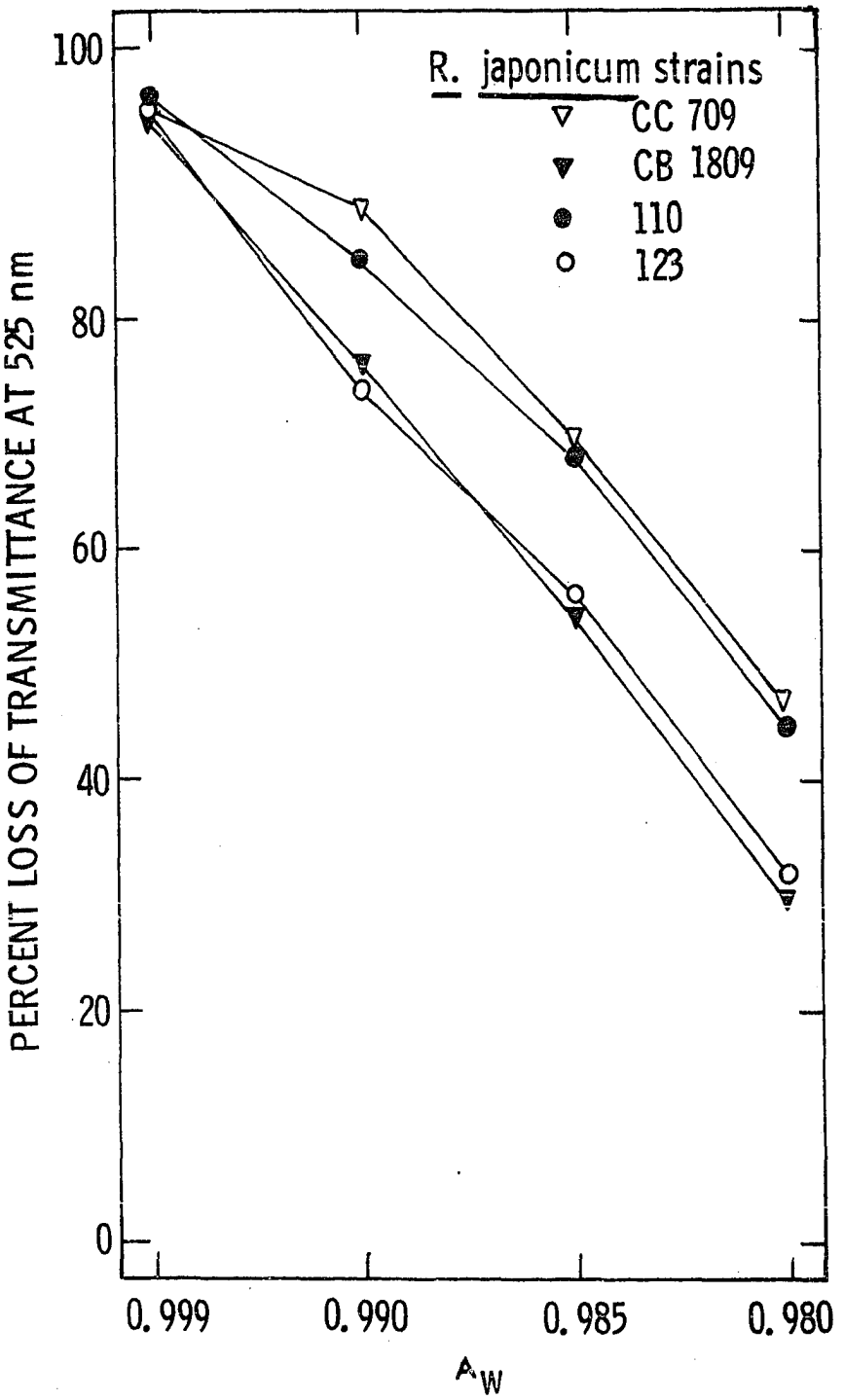
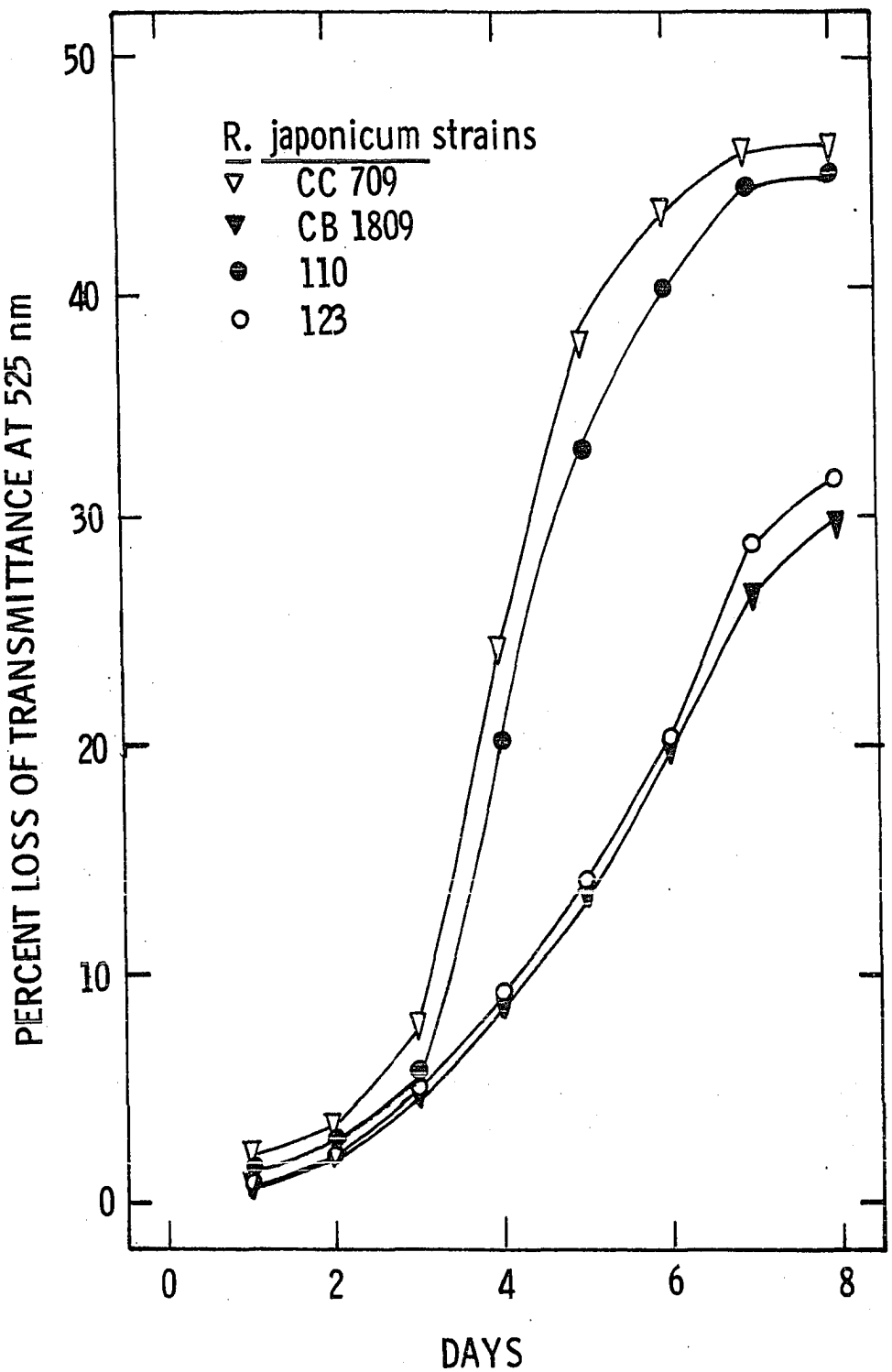

R. japonicum strains
CC 709
CB 1809
110
123
PERCENT LOSS OF TRANSMITTANCE AT 525 nm
100
80
60
40
20
0
0.999
0.990
0.985
0.980
A_W

Figure 12. The growth curves of R. japonicum strains in yeast extract broth adjusted with glycerol to a 0.980 water activity


R. japonicum strains
CC 709
CB 1809
110
123
PERCENT LOSS OF TRANSMITTANCE AT 525 nm
DAYS
0
10
20
30
40
50
0
2
4
6
8

introduced into soil. Klein and Casida (1967) found a population decrease of 6 log units in 24 days when Eschericia coli was added to soil. Dickey (1961) found a decrease in 8 weeks of almost 4 log units in the cell density of Agrobacterium tumefaciens when inoculated into soil. Working with R. meliloti M_3V_2-S in desiccated soil, Danso and Alexander (1974) reported a decrease in numbers of over 1 log unit within 8 weeks. Marshall (1964) observed that the fast-growing rhizobia were more susceptible to desiccation in sandy soils than the slow-growing species. Similar results were also obtained by Bushby and Marshall (1977a). However in the present study, strain differences were observed in the same species (R. japonicum) when members were introduced into dried soils.

Improving the Survivability of R. japonicum Strains

Survival of all four R. japonicum strains after growth at A_w 0.999 was compared with survival after growth on media adjusted to lowered A_w values, a treatment which presumably increased bacterial internal osmotic pressures. The cells were collected and washed three times with phosphate buffer adjusted to the same A_w as the growth medium. After adjusting the cell suspensions to the same turbidity, 1 ml was added to 6 g sterile sand contained in 35-mm diameter petri dishes. The dishes were incubated in a desiccator over $CaCl_2$ at 28°C, and were removed at regular intervals for viable counts using

YEM agar. Results revealed that the persistence of R. japonicum strains 110, 123, and CC709 was almost the same whether grown in basal or low-water activity media. Results with strain CB1809 showed a small increase in survival as a result of prior growth on media of lower water activity (Table 21). However, Skujins and McLaren (1967) measured the reaction rate of lypholized urea-urease mixtures under different relative humidities. They showed that at 100% relative humidity, there was a maximum hydrolysis of urea in about 2 hours. With decreasing humidity, the rate declined and there was no measurable release of $C^{14}O_2$ below 60% relative humidity. To determine the effect of water activity on nitrogen fixation, a comparison was made of nitrogen fixing activity of rhizobia grown at A_w 0.999 with the activity of cells grown at A_w 0.985. Nitrogenase activity was too variable within the same treatment to distinguish differences; however, cells grown at lower A_w level still had considerable nitrogenase activity as evaluated by acetylene reduction (Table 22).

Attempts to improve the ability of these four R. japonicum strains to withstand desiccation by growth at low A_w values were not successful, except for slight increases with strain CB1809. Chen and Alexander (1973) grew an unidentified Rhizobium sp. at low A_w values and reported increased survival when subjected to desiccation. Boylen (1973) was unable to select species of Arthrobacter for

Table 21. Effect of water activities of growth media on survival of desiccated R. japonicum strains

R. japonicum strain	A_w	Log count g^{-1} sand at various days[a]					
		0	1	5	7	11	25
110	0.999	8.00	7.57	5.49	5.15	4.92	4.54
	0.985	8.32	7.56	5.66	5.26	5.31	4.79
123	0.999	7.97	7.15	4.70	4.04	3.34	3.30
	0.985	8.04	6.91	4.53	4.00	2.90	2.77
CC709	0.999	8.22	7.14	6.36	5.68	5.04	4.94
	0.985	8.41	7.11	6.38	5.20	4.65	4.32
CC1809	0.999	7.90	5.23	3.63	2.85	2.18	2.08
	0.985	7.90	5.60	3.78	3.08	2.93	2.78

[a]Average of triplicates.

increased resistance to desiccation. Recently, Bushby and Marshall (1977a) reported that the survival in desiccated sandy soil was not influenced by prior growth of R. meliloti and slow-growing species in media of low water activity.

Water Adsorption Isotherms

Dialyzing rhizobial suspensions prior to water adsorption studies caused a change in the slope of the isotherm (Figure 13). Addition of Triton X-100 as a detergent, however, did not affect the slope of the isotherm. The variation in the isotherms between dialyzed and undialyzed suspensions was presumably due to the presence of salts in the suspensions. Bull and Breese (1970) and Gal and Bankay (1971) reported

Table 22. Effect of media A_w on acetylene reduction of R. japonicum strains

Strain	Media A_w	Activity[a] ± SD
110	0.999	50.6 ± 29.5
	0.985	44.3 ± 26.4
123	0.999	46.2 ± 24.2
	0.985	37.8 ± 22.1
CC709	0.999	49.7 ± 26.5
	0.985	52.2 ± 30.4
CB1809	0.999	47.7 ± 28.9
	0.985	40.8 ± 24.5

[a]ppm ethylene produced/hr/plant.

that sodium chloride bound to protein produces a characteristic change in water vapor sorption. Walker et al. (1973) found that the isotherm for Myosin B in which the salt (KCl) was removed by dialysis exhibited the normal sigmoid shape. Bushby and Marshall (1977b) found that dialyzed bacterial samples adsorbed more water per gram than undialyzed samples. Therefore, all isotherms of R. japonicum strains used in this study were performed on dialyzed suspensions with the addition of 1% Triton X-100.

Time studies showed that two hours were sufficient for equilibrium (Figure 14), but at least three hours were allowed to assure equilibrium at each water activity in this study.

Adsorption isotherms were conducted at 40°C by using the highly evacuated apparatus previously described. The results

Figure 13. Water adsorption isotherms at 40°C for R. japonicum strain 123 with different pre-treatments

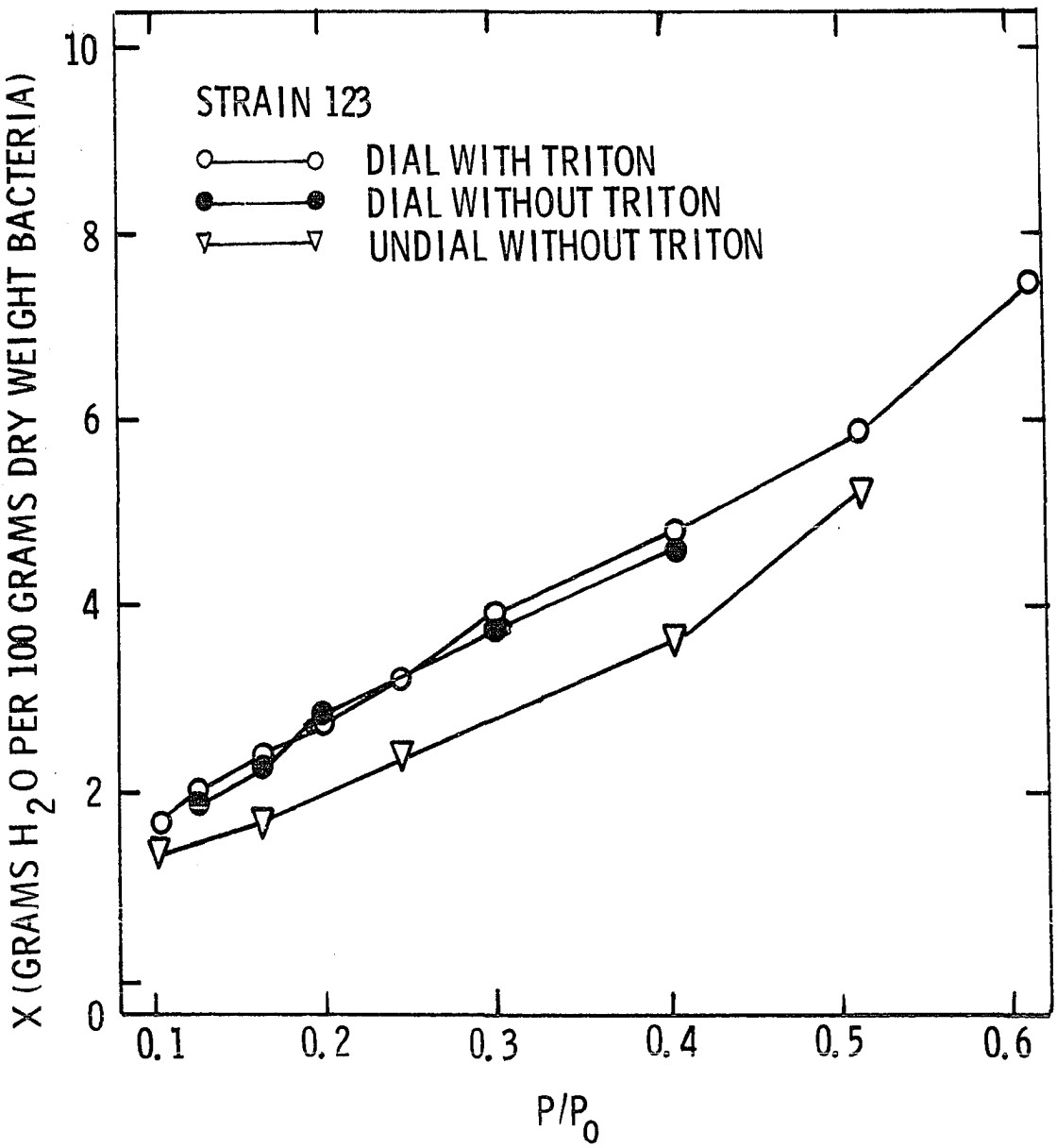
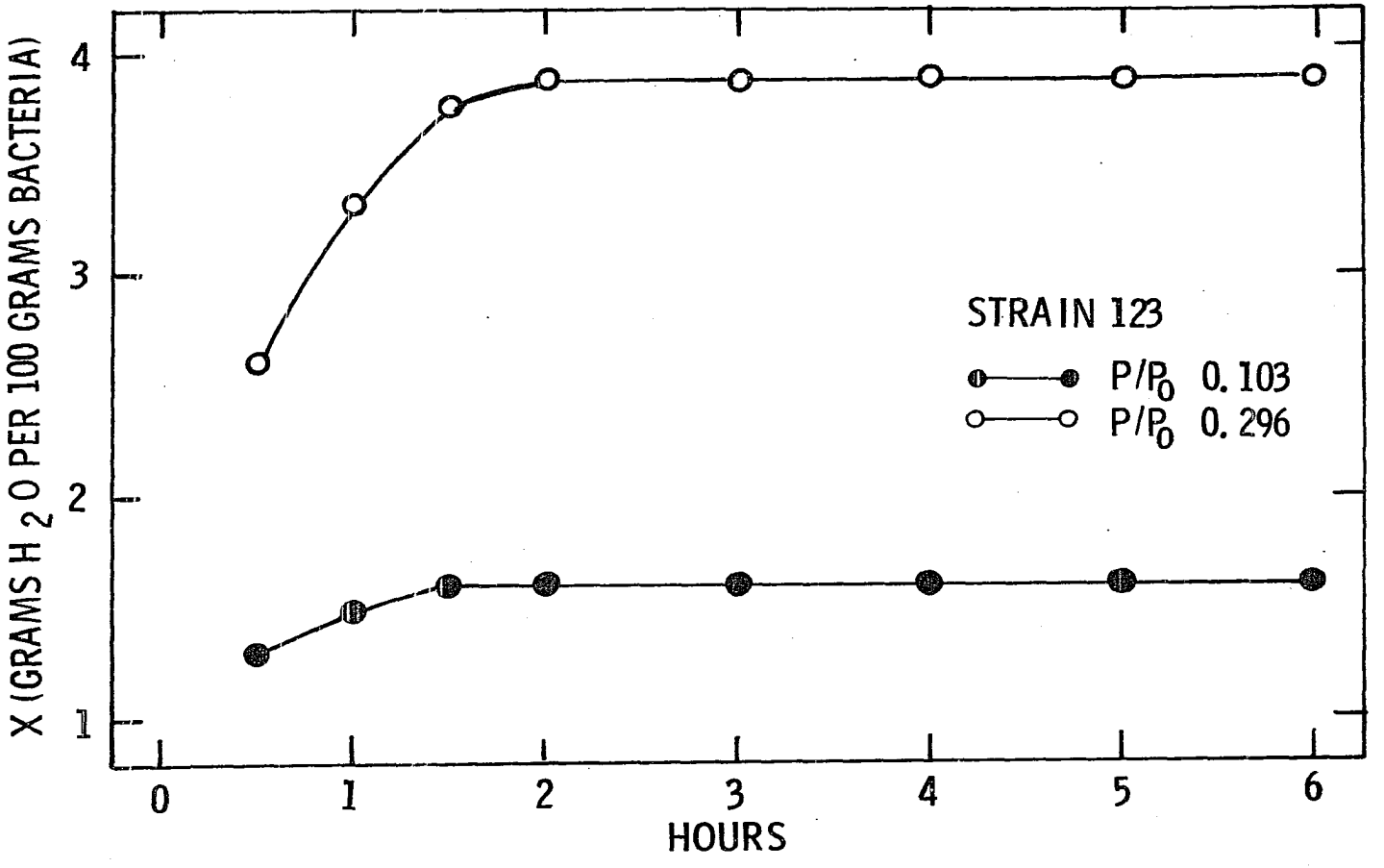
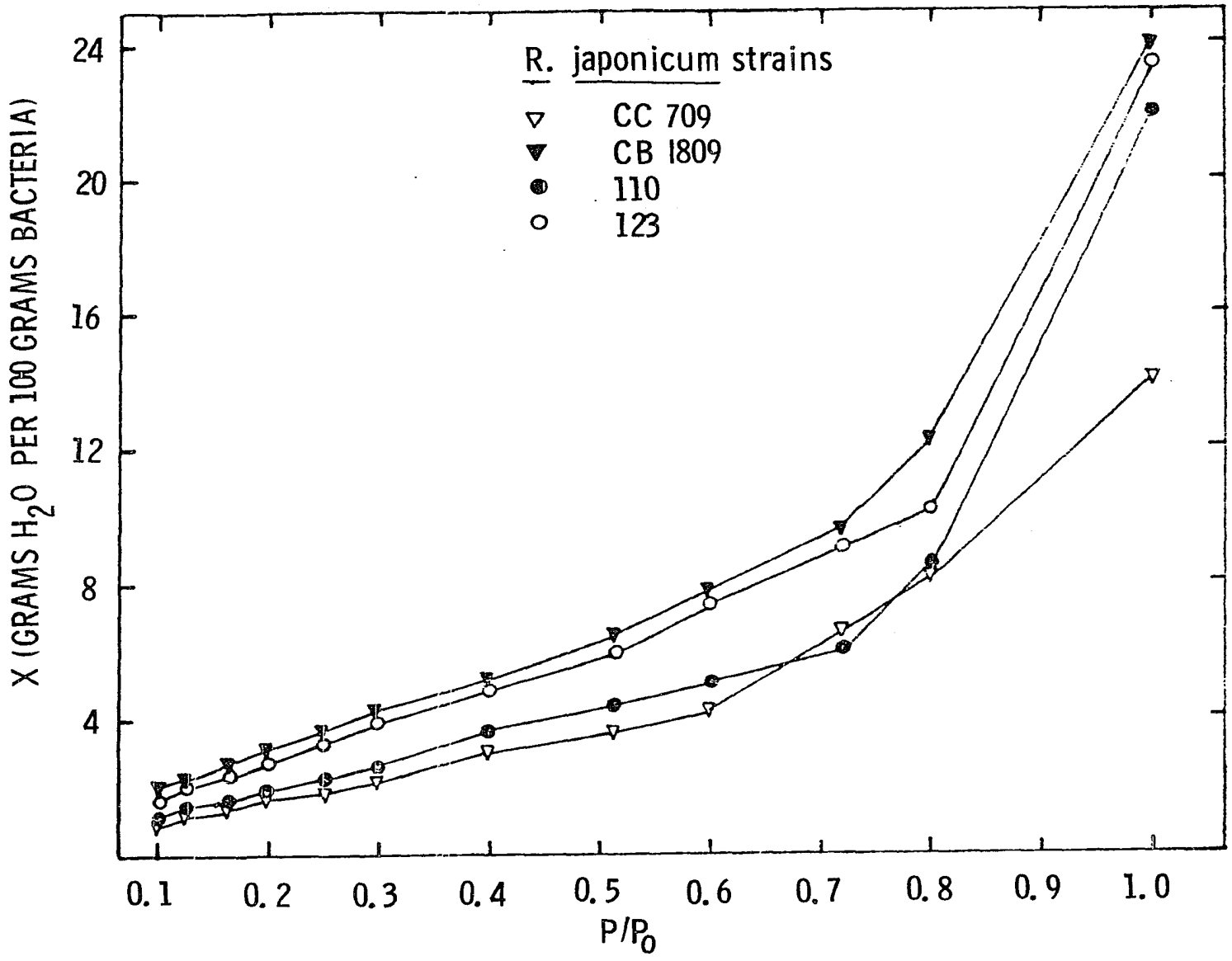

STRAIN 123
DIAL WITH TRITON
DIAL WITHOUT TRITON
UNDIAL WITHOUT TRITON
X (GRAMS H2O PER 100 GRAMS DRY WEIGHT BACTERIA)
P/P0
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.5
0.6

Figure 14. Effect of time on the equilibration of bacterial samples in the water adsorption chamber


X (GRAMS H 2O PER 100 GRAMS BACTERIA)
4
3
2
1
0
1
2
3
4
5
6
HOURS
STRAIN 123
P/P0 0.103
P/P0 0.296

indicate that R. japonicum strains 123 and CB1809 adsorbed more water than R. japonicum strains 110 and CC709 at all water activities (Figure 15). The relationship between groups was most evident at the lower relative humidities. By comparing these four strains to their susceptibility to desiccation, results indicate a definite correlation was present. Strains 123 and CB1809 were more susceptible to desiccation than strains 110 and CC709 in soils, sand, and broth cultures with low water activity, but retained more water at a given relative humidity. Thus, when limited quantities of water are available for growth under dry conditions, perhaps in strains 123 and CB1809 the water becomes associated with the cell surface and is not utilized internal to the cell membrane for metabolic functions. Since the cells were lypholized prior to water sorption studies, it is assumed that most of the water sorbed was retained on the cell surface. Mellon et al. (1947) compared the water adsorption of casein and of benzolylated casein (blocking of amino groups) and concluded that the free amino groups in amino acid residue side chains play an important role in the adsorption of vapor. Furthermore, Marshall (1967) suggested that the surfaces of the slow-growing rhizobia (less sensitive to desiccation) contain only carboxyl groups while the fast-growing rhizobia (sensitive to desiccation) contain some amino groups along with the predominant carboxyl groups. Marshall (1969) reported differences in the surface ionogenic groups within the same species.

Figure 15. Water adsorption isotherms at 40°C for different R. japonicum strains


R. japonicum strains
CC 709
CB 1809
110
123
X (GRAMS H_2O PER 100 GRAMS BACTERIA)
24
20
16
12
8
4
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
P/P_0

He reported that R. trifolii TA1, which is a fast-growing rhizobia, contained only carboxyl groups normally found in the slow-growing rhizobia. This strain was found to persist in sandy soils. Recently, Bushby and Marshall (1977b) found that greater amounts of water were retained at lower relative vapor pressures by the fast-growing rhizobia (sensitive to desiccation) than by the slow-growing rhizobia (less sensitive to desiccation). They related the differences in susceptibility to desiccation between the two groups of rhizobia to the different amounts of water retained at any relative vapor pressure.

Differences obtained in the amounts of water absorbed between R. japonicum strains 123 and CB1809 and strains 110 and CC709 and their susceptibility to desiccation agree with results obtained by Bushby and Marshall (1977b) in comparing fast and slow growing rhizobia. It seems reasonable that the surface ionogenic groups of these strains might also be different.

Bacterial Cell Morphology and Susceptibility to Desiccation

Lamanna and Mallette (1965) pointed out that under condition of desiccation, bacterial cells decrease their surface to volume ratio and become more spherical. Casida (1971) found that the dormant bacterial population of unamended soil appeared to be composed mainly of coccoid and coccoid-rod

cells. Therefore, to test the hypothesis that differences in size of R. japonicum strains affected their susceptibility to desiccation, total surface areas were measured. Four-day old suspensions were prepared and the surface areas were measured by phase-contrast microscopy. Results revealed that there were no significant differences in surface areas between the strains (Table 23).

Table 23. Surface area of R. japonicum strains measured by phase-contrast microscopy

R. japonicum strain	Number of measurements	Surface area (μm^2)
110	28	5.29 ± 0.387[a]
123	27	5.10 ± 0.329
CC709	26	4.93 ± 0.301
CB1809	26	4.99 ± 0.327

[a]Standard error of the means.

Application of the B.E.T. Theory

The B.E.T. theory (Brunauer, Emmett, and Teller, 1938) has been applied to calculate the surface area of many substances. It was applied by Bull (1944) for proteins, Orchiston (1953) for soils, Bateman et al. (1962) for Serratea marcescens, and by Bushby and Marshall (1977b) for slow- and

fast-growing rhizobia.

The B.E.T. equation states that:

$$\frac{P/P_o}{X(1-P/P_o)} = \frac{(C-1)}{X_m.C} P/P_o + \frac{1}{X_m.C}$$

where X is the amount of water vapor sorbed per 100 g dry weight at a pressure P, P_o is the saturated vapor pressure, X_m is the amount of water required to form a monolayer over the adsorbing surface, and C is a constant from which the heat of adsorption for the monolayer can be obtained.

When $P/P_o/[X(1-P/P_o)]$ is plotted against P/P_o, a straight line is obtained. Bull (1944) found, in general, that straight lines were obtained for protein sorption between P/P_o of 0.05 to 0.5. Both below and above these relative vapor pressures, departure from a linear relation occurred. Figure 16 shows the B.E.T. plots for R. japonicum strains 110, 123, CC709, and CB1809. Since the slopes of these lines are given by $(C-1)/X_m.C$ and their intercepts by $1/X_m.C$, the two constants X_m and C can be determined. From the lines of best fit by the method of least squares, the constants X_m and C were calculated (Table 24).

If we assume (Orchiston, 1953) that a water molecule adsorbed by the cell surface occupies 10.5 sq. Angstrons, the specific surface area of the cell can be calculated from X_m as follows:

$$\frac{X_m(\text{g } H_2O/100 \text{ g bact.})}{\text{Mol. wt. of } H_2O} = \text{Moles } H_2O$$

Figure 16. The B.E.T. plots for R. japonicum strains: ● strain 110, ○ strain 123, ▽ strain CC709, and ▼ strain CB1809

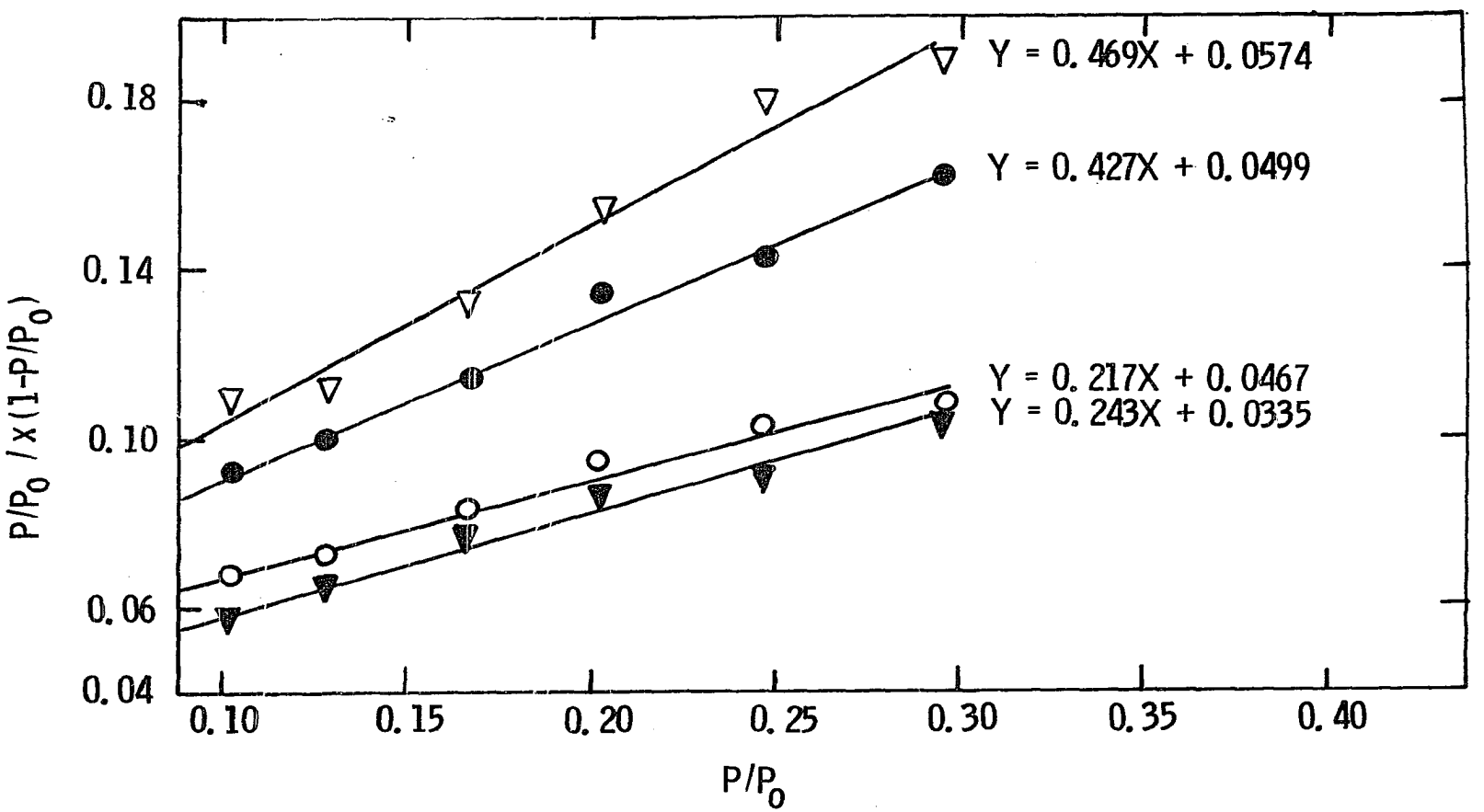

Y = 0.469X + 0.0574
Y = 0.427X + 0.0499
Y = 0.217X + 0.0467
Y = 0.243X + 0.0335
0.18
0.14
0.10
0.06
0.04
0.10
0.15
0.20
0.25
0.30
0.35
0.40
P/P0 /x(1-P/P0)
P/P0

Table 24. Brunaur et al. (1938) constants and calculated surface areas of R. japonicum strains

R. japonicum strain	X_m [a]	C [b]	Surface area (m^2g^{-1})
110	2.10	9.56	73.77
123	3.79	5.65	133.15
CC709	1.90	9.17	66.75
CB1809	3.63	8.25	127.53

[a] X_m is the amount of water required to form a monolayer over the adsorbing surface.

[b] C is a constant from which the heat of adsorption for the monolayer can be obtained.

Number of H_2O molecules/100 g bact. = Moles H_2O x Avogadro no. Assuming one H_2O molecule occupies 10.5 sq. Å, then,

Surface area = 10.5 x number of H_2O molecules.

The specific surface areas in square meters per gram were computed for the various R. japonicum strains and are presented in column 4 of Table 24. Results by this method show that the average surface area available for water sorption for R. japonicum strains 123 and CB1809 was approximately twice that available in strains 110 and CC709. This indicates that greater available surface areas were associated with increased susceptibility of R. japonicum strains to desiccation and was not related to the total surface area of these strains.

Changes in Rhizobia Surface Free Energy

Changes in free energy when solid or porous materials adsorb water vapor have been determined by application of Bangham's (1937) free energy equation to isotherm data (Bull, 1944; Sharma et al., 1969; Bushby and Marshall, 1977b). This equation can be expressed as:

$$\Delta F = \frac{-RT}{MA} \int_{o}^{1} \frac{X}{P/P_o} \, d(P/P_o)$$

when R is the gast constant, T is the absolute temperature, M is the molecular weight of water, A is the specific surface area of the adsorbing material, X is the weight of water sorbed per 100 g dry weight, P/P_o is the relative vapor pressure, and ΔF is the change in free energy involved with wetting.

In order to integrate this equation, $X/(P/P_o)$ was plotted against P/P_o and the area under the curve was measured by using a planometer (Table 29, Appendix). When this area was multiplied by RT/M, the free energy change was obtained. Values for $A\Delta F$ were determined for various P/P_o levels of each R. japonicum strain studied (Table 25).

Results at all relative vapor pressures indicates that the surface energies of R. japonicum strains 123 and CB1809 were greater than strains 110 and CC709. These results agree with those of Bushby and Marshall (1977b) when a comparison

Table 25. Changes in surface energy of different R. japonicum strains at various relative vapor pressures

	-AΔF (cal./100 g dry wt.)			
P/P_o	110	123	CC709	CB1809
0.203	106.8	150.9	91.8	174.5
0.296	198.6	283.7	160.3	317.8
0.404	290.0	408.4	236.0	447.6
0.512	380.3	525.5	308.8	571.2
0.609	462.4	645.0	378.2	696.0
0.721	546.0	767.2	445.8	830.9
0.805	638.6	891.3	526.4	957.4
1.000	809.1	1240.8	751.2	1345.5

was made between the desiccation-sensitive group of rhizobia and their changes in surface energies.

SUMMARY AND CONCLUSIONS

Survival of four R. japonicum strains, two USDA strains 110 and 123 and two Australian strains CC709 and CB1809, was determined in three gamma sterilized soils: Hayden sandy loam, Nicollet loam, and Okoboji silty clay. Antibiotic-resistant strains were cultured in order to evaluate strain survival following soil addition and to study factors affecting their survival.

Growth after 7 weeks incubation showed that soils, strains, treatments, and time of incubation significantly affected the survival. Treatments consisted of moisture at field capacity and dried over $CaCl_2$, temperature at 27 and 36°C, and salt levels at 0, 0.3, and 0.7%. The moisture content of the soil played a dominant role in the survivability of all four R. japonicum strains. Temperature and salt also played statistically significant roles, but to a lesser degree than moisture. The most significant decline in Rhizobium sp. numbers occurred when soils were desiccated and incubated at 36°C. Decline also occurred when soils were desiccated and incubated at 27°C, but to a lesser degree than at 36°C. Statistical analysis of the salt effect showed that the viable count of Rhizobium sp. decreased linearly as the salt was increased from 0 to 0.7%. This effect was greater in desiccated soils than in moist soils.

Rhizobium japonicum strains were found to significantly

differ in their susceptibility to desiccation in a given soil. Generally, strain 110 was similar to strain CC709, and strain 123 was similar to strain CB1809 at the 0.01 confidence level. However, both strains 110 and CC709 were significantly different at the 0.01 level from strains 123 and CB1809. Additionally, to verify these results, comparisons were made of the survivability of R. japonicum strains grown in sterilized sand desiccated over $CaCl_2$ and in YEB adjusted with glycerol to lower A_w levels. Strains sensitivities to lowered water potentials were in general agreement with those obtained in desiccated soils. Attempts to improve the ability of these strains to withstand desiccation in sterilized sand by prior growth in YEB adjusted to low A_w values were not successful, except for small increases with strains CB1809.

Effects of media water activity on nitrogen fixation was determined. Nitrogenase activity was too variable within the same treatment to distinguish differences; however, cells grown at low A_w values still had considerable nitrogenase activity.

Water adsorption isotherms showed that the more susceptible strains to desiccation, 123 and CB1809, compared with the less susceptible strains, 110 and CC709, were related to the different amounts of water retained by these strains at a given relative vapor pressure. Strains 123 and CB1809 adsorbed more water than strains 110 and CC709 at all water activities. The B.E.T. theory (Brunauer, Emmett, and Teller,

1938) was applied to the data from the water adsorption isotherms to calculate the active surface areas. Results showed that the higher retention of water by the more susceptible strains at a given relative vapor pressure was related to greater availability of adsorptive surface area. Values for change in surface energy ($A\Delta F$) were obtained for various relative vapor pressures, and the results show that, at all relative vapor pressures, the surface energies of susceptible strains were greater than surface energies of less susceptible strains.

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APPENDIX

Table 26. Percentage loss of transmittance of R. japonicum strains grown in YEB with various water activities

R. japonicum strain	A_w	Time, days							
		1	2	3	4	5	6	7	8
110	0.999	19.5	65.3	75.9	82.5	91.6	92.9	94.0	96.0
	0.985	3.4	7.0	15.6	35.6	51.3	59.3	64.2	68.2
	0.980	1.7	2.9	5.7	20.5	32.9	40.0	44.5	45.0
123	0.999	27.3	53.4	75.0	87.5	98.6	93.7	94.5	95.0
	0.985	2.3	5.7	9.1	17.0	29.0	48.1	54.0	56.8
	0.980	1.1	2.3	5.6	9.2	14.3	20.5	29.0	31.8
CC709	0.999	22.6	56.8	82.4	89.4	92.4	92.9	94.0	96.0
	0.985	3.4	14.8	35.0	51.1	56.8	60.2	65.9	69.3
	0.980	2.3	3.4	7.5	23.9	57.5	43.2	45.5	46.0
CB1809	0.999	20.4	49.3	72.3	87.0	88.0	91.6	92.8	94.0
	0.985	2.3	5.5	8.7	15.6	28.9	45.9	52.8	54.4
	0.980	1.1	2.3	5.1	8.7	13.5	20.1	26.5	29.5

Figure 17. Calibration curve of silica spring used to measure equilibration of rhizobial cells with water vapor

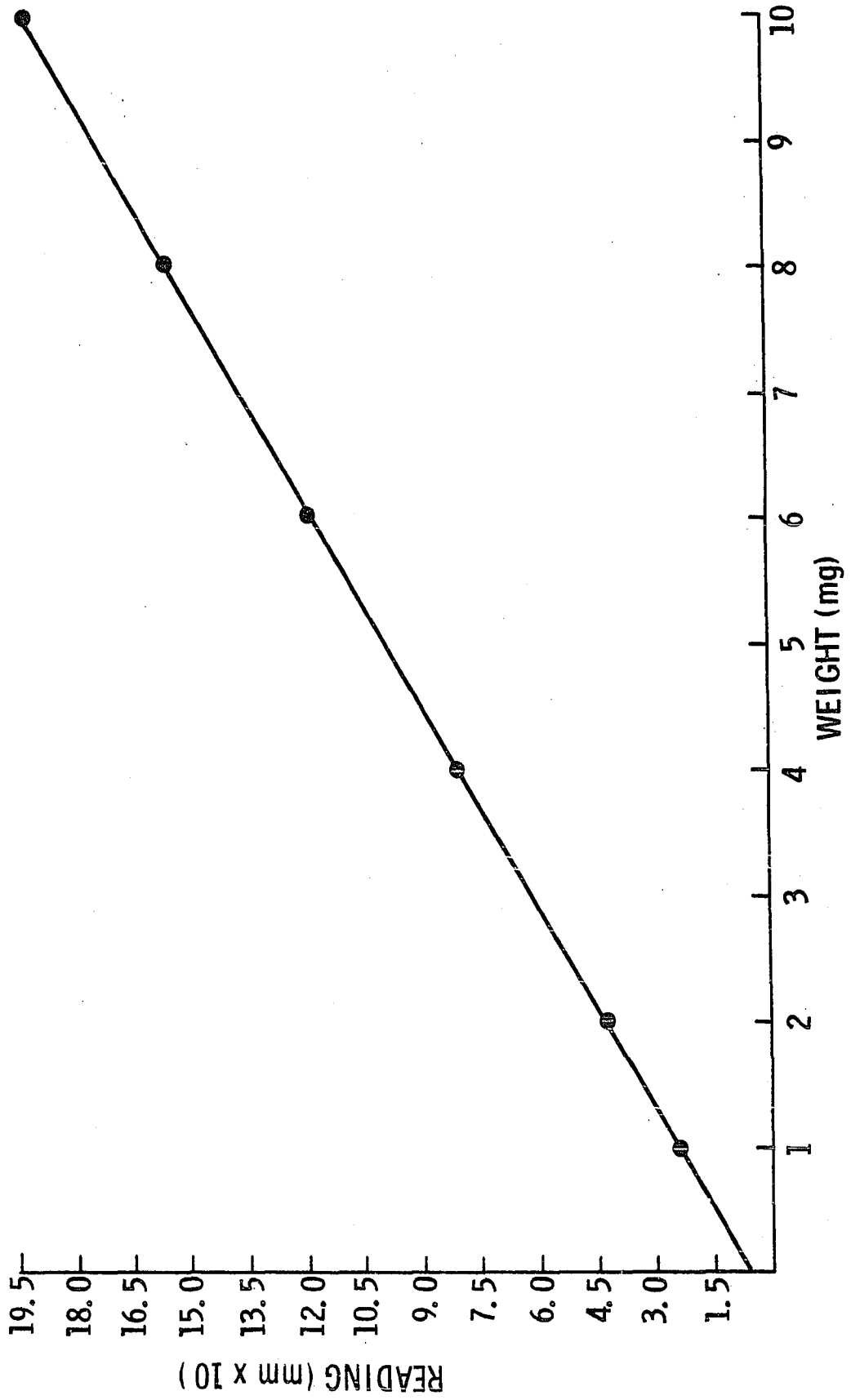
READING (mm x 10)
19.5
18.0
16.5
15.0
13.5
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
WEIGHT (mg)
1
2
3
4
5
6
7
8
9
10

Table 27. Pressure of aqueous vapor (mm H_g) over water in the water container at the bottom of the water sorption apparatus (cited from Handbook of Chemistry and Physics, 1973-1974, pp. D159)

Temp °C	0.0	0.2	0.4	0.6	0.8
3	5.685	5.766	5.848	5.931	6.015
6	7.013	7.111	7.209	7.304	7.411
10	9.209	9.333	9.458	9.585	9.714
13	11.231	11.379	11.528	11.680	11.833
16	13.634	13.809	13.987	14.166	14.347
19	16.477	16.685	16.874	17.105	13.319
24	22.377	22.648	22.922	23.198	23.476
28	28.349	28.680	29.015	29.354	29.697
31	33.695	34.082	34.471	34.864	35.261
34	39.898	40.394	40.796	41.251	41.710
36	44.563	45.054	45.549	46.050	46.556
38	49.692	50.231	50.774	51.323	51.879
40	55.324	55.910	56.510	57.110	57.720

Table 28. Comparison of the amounts of water adsorbed by unlyophilized egg albumin obtained by Bull (1944) and in apparatus used in this study

Water activity (P/P_o)	Grams H_2O per 100 grams egg albumin	
	Bull (1944)	Apparatus[a]
0.1	3.63	3.70
0.2	5.50	5.40
0.3	7.14	-
0.4	8.84	8.50
0.5	10.40	10.10
0.6	12.12	11.30
0.7	14.35	14.00
0.8	18.00	-

[a]Average of two replicates.

Table 29. Area under the curve (cm^2) as measured by a planometer by plotting x $(P/P_o)^{-1}$ against P/P_o for calibrating the changes in cells surface free energy

Water activity (P/P_o)	*R. japonicum* strains			
	110	123	CC709	CB1809
0.203	3.54	5.00	3.04	5.78
0.296	6.58	9.40	5.31	10.53
0.404	9.61	13.53	7.82	14.83
0.512	12.60	17.41	10.23	18.93
0.609	15.32	21.37	12.53	23.06
0.721	18.09	25.42	14.77	27.20
0.805	21.16	29.53	17.44	31.72
1.000	26.74	41.11	24.89	44.58

Table 30. Water adsorption by lyophilized R. japonicum strain 110 at various water activities

Water activity (P/P_o)	Weight of sample= 0.235 g		Weight of sample= 0.0196 g		
	Reading (mmx10)	H_2O sorbed (mg)	Reading (mmx10)	H_2O sorbed (mg)	G H_2O/100 g dry wt bact. (average)
0.103	1.140	0.31	1.020	0.24	1.25
0.127	1.170	0.33	1.080	0.29	1.45
0.166	1.350	0.42	1.170	0.33	1.75
0.203	1.350	0.42	1.290	0.39	1.90
0.246	1.530	0.54	1.380	0.45	2.30
0.296	1.695	0.61	1.725	0.51	2.60
0.404	2.781	0.92	1.720	0.73	3.80
0.512	2.400	0.99	2.130	0.84	4.25
0.609	2.700	1.15	2.475	1.02	5.05
0.721	3.165	1.41	2.745	1.18	6.00
0.805	4.350	2.02	3.525	1.63	8.45
1.000	13.050	6.67	10.950	5.57	23.80

Table 31. Water adsorption by lyophilized R. japonicum strain 123 at various water activities

Water activity (P/P_o)	Weight of sample =0.0188 g		Weight of sample =0.0206 g		G H_2O/100 g dry wt bact. (average)
	Reading (mmx10)	H_2O sorbed (mg)	Reading (mmx10)	H_2O sorbed (mg)	
0.103	1.125	0.30	1.275	0.37	1.70
0.127	1.200	0.34	1.380	0.45	2.00
0.166	1.350	0.43	1.545	0.52	2.40
0.203	1.425	0.47	1.680	0.60	2.70
0.246	1.500	0.56	1.845	0.70	3.20
0.296	1.875	0.71	2.100	0.82	3.90
0.404	2.250	0.90	2.400	0.99	4.80
0.512	2.580	1.09	2.850	1.24	5.90
0.609	3.150	1.39	3.480	1.57	7.50
0.721	3.600	1.62	4.050	1.85	8.80
0.805	4.110	1.88	4.500	2.11	10.20
1.000	8.295	4.14	9.000	4.53	22.00

Table 32. Water adsorption by lyophilized R. japonicum strain CC709 at various water activities

Water activity (P/P_o)	Weight of sample =0.0229 g		Weight of sample =0.0247 g		G H_2O/100 g dry wt bact. (average)
	Reading (mmx10)	H_2O sorbed (mg)	Reading (mmx10)	H_2O sorbed (mg)	
0.103	0.975	0.23	1.065	0.27	1.05
0.127	1.080	0.27	1.230	0.35	1.30
0.166	1.200	0.34	1.275	0.37	1.50
0.203	1.320	0.39	1.305	0.40	1.65
0.246	1.350	0.41	1.395	0.45	1.80
0.296	1.500	0.50	1.575	0.54	2.20
0.404	1.920	0.73	1.950	0.74	3.10
0.512	2.070	0.80	2.175	0.86	3.50
0.609	2.340	0.96	2.505	1.04	4.20
0.721	2.415	1.08	2.775	1.19	4.75
0.805	3.480	1.56	4.275	1.98	7.90
1.000	6.600	3.21	6.600	3.21	13.00

Table 33. Water adsorption by lyophilized R. japonicum strain CB1809 at various water activities

Water activity (P/P_o)	Weight of sample =0.0189 g		Weight of sample =0.0205 g		G H_2O/100 g dry wt bact. (average)
	Reading (mmx10)	H_2O sorbed (mg)	Reading (mmx10)	H_2O sorbed (mg)	
0.003	1.245	0.36	1.350	0.43	2.00
0.127	1.350	0.42	1.380	0.49	2.30
0.166	1.380	0.49	1.545	0.53	2.60
0.203	1.650	0.57	1.770	0.64	3.05
0.246	1.815	0.66	1.965	0.76	3.60
0.296	1.950	0.74	2.115	0.84	4.00
0.404	2.325	0.94	2.520	1.04	5.00
0.512	2.880	1.21	3.000	1.31	6.40
0.609	3.270	1.44	3.540	1.60	7.70
0.721	3.675	1.68	4.050	1.87	9.00
0.850	4.815	2.27	5.130	2.46	12.00
1.000	9.000	4.50	9.750	4.90	23.90